In re Application of: Michal Amit et al.
Serial No.: 10/581,455
Filed: June 1, 2006
Office Action Mailing Date: March 2, 2009

Examiner: TON, Thaian N
Group Art Unit: 1632
Attorney Docket: 32059

REMARKS

Reconsideration of the above-identified application in view of the amendments above and the remarks following is respectfully requested.

Claims 52, 55-75 and 78-84 are in this Application. Claims 61-63, 65-73 have been withdrawn from consideration. Claim 64 has been withdrawn and currently amended. Claim 59 has been objected to. Claim 78 has been rejected under 35 U.S.C. § 112. Claims 52, 55, 56, 58-60, 74, 75, 78-80 have been rejected under 35 U.S.C. § 102. Claims 52, 55, 56, 57, 58-60, 74, 75, 78-80, 81, 82-84 have been rejected under 35 U.S.C. § 103. Claims 52, 55-60 and 78 are amended herewith.

Amendments To The Claims

Claim Objection

The Examiner objected to claim 59 because the word "isolated" was misspelled.

Applicant has corrected the error, to thereby overcome Examiner's objection.

35 U.S.C. § 112 Rejections

The Examiner rejected claim 78 under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention. Specifically, the Examiner questioned the antecedent basis for the limitation "said stem cell".

Applicant has amended claim 78 to recite *"said stem cell line"* (Emphasis added), which is recited in step (a) of claim 74, to thereby overcome the Examiner's rejection.

In view of the above claims amendments, Applicants believe to have overcome the 35 U.S.C. § 112, second paragraph rejections.

35 U.S.C. § 102 Rejections

Amit et al. 2003

The Examiner has rejected claims 52, 55, 56, 58-60, 74, 75, 78-80 under 35 U.S.C. 102 (a) as being anticipated by *Amit et al.* (Chapter 7: Subcloning and Alternative Methods for the Derivation and Culture of Human Embryonic Stem Cells from Human Embryonic Stem Cells, January 1, 2003, pp. 127-144). Specifically, the Examiner states that *Amit et al.*, teach a human ES cell line (J-3) heterozygous for the W128X mutation that has been in continuous culture for over 116 passages and had normal karyotype; that producing human ES cell lines that harbor different genetic defects and following the expression of the disease during differentiation can be used to develop drugs or gene therapy to treat these genetic diseases. The Examiner's rejection is respectfully traversed.

Applicants respectfully request the withdrawal of this rejection on the grounds that the *Amit et al.* reference is not prior art relative to the instant application.

As shown by the attached Declaration of Joseph Itskovitz Eldor under 37 CFR 1.132, Hanna Segev and Dorit Manor were identified as co-authors of the above publication for their clinical efforts conducted under the direct supervision and direction of Joseph Itskovitz Eldor and are therefore not inventors.

As such, the *Amit et al.* reference cannot be used as prior art against the instant application since it is inventors' own work which was published within the one year grace period preceding the filing date of U.S. Provisional Application No. 60/525,883, filed on December 1, 2003. In re Katz, 687 F. 2d 450, 215 USPQ 14 (CCPA 1982). Withdrawal of the rejection is respectfully requested.

Zwaka et al. 2003

The Examiner rejected claims 52, 55, 56, 58-60, under 35 U.S.C. 102 (a) as being anticipated by Zwaka et al. (Nature Biotechnology, 21:319-321, March 2003). Specifically, the Examiner states that Zwaka et al., teach homologous recombination in human ES to successfully target the HPRT1 gene, and that HPRT1 deficiency in humans results in Lesch-Nyhan syndrome.

Applicants respectfully traverse this rejection. However, in order to simplify the issues, Applicants attach herewith a Declaration under 37 C.F.R. §1.131 by

Applicants Dr. Michal Amit and Prof. Joseph Itskovitz Eldor in which they show a reduction to practice of the claimed invention (in the form of the *Amit et al.*) prior to the publication date of the Zwaka et al. publication, *i.e.*, February 2003.

In view thereof, the reference of Zwaka et al., 2003 is not prior art reference relative to the instant application, and the rejection of the claims over Zwaka et al. is no longer valid and should be withdrawn.

US 2006/0128018

The Examiner rejected claims 52, 55, 56, 58-60, under 35 U.S.C. 102 (e) as being anticipated by PGPub US 2006/0128018 (Zwaka et al., Published June 15, 2006; priority document filed February 7, 2003). Specifically, the Examiner states that the US 2006/0128018 application teaches targeted gene delivery by homologous recombination to human ES cells, targeting the HPRT gene and disruption of this locus; that ES cells containing a specific genetic modification can be differentiated and used for screening methods.

Applicants respectfully traverse this rejection. However, in order to simplify the issues, Applicants attach herewith a Declaration under 37 C.F.R. §1.131 by Applicants Dr. Michal Amit and Prof. Joseph Itskovitz Eldor in which they show a reduction to practice of the claimed invention (in the form of the Amit et al. 2003, Chapter 7 publication) prior to the publication date of the Zwaka et al. document (US 2006/0128018), *i.e.*, February 2003.

In view thereof, PGPub US 2006/0128018 is not prior art reference relative to the instant application, and the rejection of the claims over PGPub US 2006/0128018 is no longer valid and should be withdrawn.

In view of the above arguments and remarks Applicants believe to have overcome the 35 U.S.C. § 102 rejections.

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35 U.S.C. § 103 Rejections

US 2006/0128018 and US 2002/0081668

The Examiner rejected claims 74, 75, 78-79, 82-83 under 35 U.S.C. 103(a) as being unpatentable over PGPub US 2006/0128018, when taken with PGPub US 2002/0081668 (published June 27, 2002, filed November 30, 2002). Specifically, the Examiner states that US 2006/0128018 teaches targeted delivery by homologous recombination to human ES cells, targeting the HPRT gene and disruption of this locus; that ES cells containing a specific genetic modification can be differentiated and used for screening methods; that after the ES cells are transfected they are permitted to differentiate by spontaneous aggregation (EBs) and that the desired differentiated cells can be identified by optical cell sorting techniques such as FACS. The Examiner further states that US 2006/0128018 does not specifically teach utilizing the ES cells in methods of identifying agents suitable for treating a disorder associated with the disease-causing mutation, however, the 2002/0081668 document teaches mutated mouse ES cells in the discovery and development of new therapeutic and diagnostic agents. The Examiner's rejection is respectfully traversed.

Applicants point out that since reduction to practice of the claimed invention (in the form of the *Amit et al.*) was prior to the filing date of US 2006/0128018, *i.e.*, prior to February 7, 2003, US 2006/0128018 cannot be considered as novelty or inventive step destroying of the instant application, either alone or in combination with 2002/0081668, which merely teaches mouse ESCs and their use for development of therapeutic and diagnostic agents, but does not teach or suggest a human ESC line harboring a disease-causing mutation and methods of using same as in the claimed invention. Withdrawal of the rejection is respectfully requested.

US 2006/0128018, US 2002/0081668 and US 2005/0054092

The Examiner rejected claim 84 under 35 U.S.C. 103(a) as being unpatentable over PGPub US 2006/0128018, when taken with PGPub US 2002/0081668 as applied to claims 74, 75, 78-79, 82-83, and further in view of PGPub US 2005/0054092. Specifically, the Examiner states that US 2006/0128018 and US 2002/0081668 do not specifically teach isolating lineage specific cells by mechanical separation of cells,

tissues or tissue-like structures contained within the embryoid body, however, the US 2005/0054092 document teaches that suspension of pPS derived cells can be further enriched with the desired characteristics, such as mechanical separation or cell sorting. The Examiner's rejection is respectfully traversed.

Applicants point out that since reduction to practice of the claimed invention (in the form of the *Amit et al.*) was prior to the filing date of US 2006/0128018, *i.e.*, prior to February 7, 2003, US 2006/0128018 cannot be considered as novelty or inventive step destroying of the instant application, either alone or in combination with 2002/0081668, which merely teaches mouse ESCs and their use for development of therapeutic and diagnostic agents, or US 2005/0054092 which merely teaches mechanical separation or cell sorting of pPS derived cells, each of which or a combination thereof does not teach or suggest a human ESC line harboring a disease-causing mutation and methods of using same as in the claimed invention. Withdrawal of the rejection is respectfully requested.

Ratcliff et al., 1992, Thomson et al., 1998, US Pat. No. 7,390,659 and Elsea et al., 2002

The Examiner rejected claims 52, 55, 56, 58-60, 74, 75, 78-80 and 82 under 35 U.S.C. 103(a) as being unpatentable over Ratcliff (Transgenic Res. 1:177-181, 1992) when taken with Thomson et al., (Science, 282:1145-1147, 1998) and US Pat. No. 7,390,659, in further view of Elsea et al., (ILAR Journal, 43:66-79, 2002). Specifically, the Examiner states that Ratcliff teach the specific disruption of the cftr gene at the endogenous locus in mouse ES cells by gene targeting; utilizing these mouse ES cells, transgenic animals can be produced to study pathophysiology and testing of new therapeutic drugs. The Examiner admits that Ratcliff does not teach human ESCs or methods of using such in *in-vitro* assays. However, the Examiner asserts that Thomson teach human ESCs, and that genetic modifications could be produced in ES cells, for reducing or combating immune rejection; that Thomson teach that human ESCs can be differentiated, and that human ESCs would be valuable in studies of development and function of tissues that differ between mice and humans, and that screens based upon the in vitro differentiation to specific lineages could identify gene targets for new drugs. The Examiner further states that US Pat.

No. 7,390,659 teaches identifying candidate agents for treating conditions associated with motor neuron degeneration using ESCs which contain a mutation in a specific gene. The Examiner states that it would have been obvious to one of ordinary skill in the art, to utilize the technology to produce specific disruptions in mouse ES cells and apply this technology to human ES cells, and then utilize the resultant cells in methods of screening agents suitable for treating a disorder such as taught by US Pat. No. 7,390,659 with a reasonable expectation of success; and that one of ordinary skills in the art would have been motivated to make this modification in view of Thomson's teachings who suggest producing genetic modifications in ESCs. The Examiner's rejection is respectfully traversed.

Applicants point out that a *prima facie* case of obviousness has not been properly set by combining the art of Ratcliff (1992) with that of Thomson (1998), Elsea (2002) and US Pat. No. 7,390,659, since in spite of Thomson's statement that *"strategies to prevent immune rejections ... need to be developed but could include ... genetically manipulating ES cells to reduce ... immune rejection"* (Thomson et al., 1998, Page 1147, left column), the teachings of Ratcliff et al. with respect to homologous recombination in mouse ES cells cannot be used without major modifications on human ESCs in order to generate human ESC lines harboring genetic mutations, even in view of Elsea et al., who merely stated that there is a need for human ESCs as a model for genetic diseases, and US Pat. No. 7,390,659, which teaches methods of inducing differentiation of embryonic stem cells.

For example, it was well known in the art that in contrast to mouse ESCs which can be subject to electroporation protocols (for introducing the targeting vector for homologous recombination), human ESCs do not survive electroporation well (see Eiges R., et al., 2001, Page 515, left column, lines 2-3; attached herewith). In addition, it was known in the art that in contrast to murine ESCs, human ES cells cannot be efficiently cloned from a single cell (see Table 1 in Page 273 of Amit et al., 2000, attached herewith). Taken together, it is conceivable that screening for rare recombination events in human ESCs is extremely more complicated than in mouse ESCs, let alone generation of human ESC lines from single ESCs having the targeted recombination.

Applicants further attach herewith a Declaration under 37 C.F.R. §1.132 by Applicant Dr. Michal Amit, an expert in the field of human embryonic stem cells, which states that protocols established for mouse ESCs cannot be used without major modifications on human ESCs because of the differences mentioned above.

Moreover, even Zwaka et al., 2003 stated that *"significant differences between mouse and human ES cells have hampered the developments of homologous recombination in human ES cells"* (Zwaka et al., 2003, Abstract). Evidently, neither Thomson et al., nor any other researcher skilled in the art of human ESCs have taught or suggested, prior to conceiving of the claimed invention, how to obtain human ESC lines which harbor genetic mutations as claimed.

Thus, it is Applicants' position that due the major differences between mouse and human ESCs, and the lack of guidance in Ratcliff et al., Thomson et al., Elsea (2002) and US Pat. No. 7,390,659 with respect to what modifications are needed for performing homologous recombination in human ESCs, one of ordinary skill in the art would not have been motivated to apply the technology of producing specific disruptions in mouse ESCs on human ESCs to thereby generate the human ESC lines carrying a disease-causing mutation in a genomic polynucleotide sequence thereof as claimed.

In sharp contrast to the genetically-modified mouse ESCs taught by Ratcliff et al., which were generated by homologous recombination, the present inventors have uncovered that human ESC lines which carry disease-causing mutations can be established from human blastocysts which are selected based on the presence of the disease-causing mutation. Thus, as shown in Example 1 of the instant application, the present inventors have identified in human blastomeres cells which carry disease-causing mutations, and further generated from such human blastomeres ESC lines which carry the disease-causing mutations in their genome (Pages 43-49 in the instant application as filed). Using the novel approach of the claimed invention human ESC lines which carry in their genome any naturally-occurring disease-causing mutation can be established and further used as disease-models for the identification of drugs suitable for treating the genetic diseases caused by the mutations.

Withdrawal of the rejection is respectfully requested.

Ratcliff et al., 1992, Thomson et al., 1998, Elsea et al., 2002 and US 2005/0054092

The Examiner has rejected claims 83-84 under 35 U.S.C. 103(a) as being unpatentable over Ratcliff (Transgenic Res. 1:177-181, 1992) when taken with Thomson et al., (Science, 282:1145-1147, 1998) in further view of Elsea et al., (ILAR Journal, 43:66-79, 2002) as applied to claims 52, 55, 56, 58-60, 74, 75, 78-80, 82 above, and further in view of PGPub US 2005/0054092. Specifically, the Examiner states that Ratcliff, Thomson and Elsea which are described above do not specifically teach isolating lineage specific cells by mechanical separation of cells or tissues within the embryoid body, however, US 2005/0054092 teaches that suspension of pPS derived cells can be further enriched with desirable characteristics, such as mechanical separation or cell sorting, such as FACS. Thus, the Examiner states that it would have been obvious for one of skill in the art to modify the methods taught by Ratcliff, Thomson and Elsea, to include a step of isolating a lineage specific cell as taught by US 2005/0054092 with a reasonable expectation of success, and that one of ordinary skill in the art would have been motivated to make this modification in order to have a purified population of cells for in vitro screening assays. The Examiner's rejection is respectfully traversed.

Applicants point out that a *prima facie* case of obviousness has not been properly set by combining the art of Ratcliff (1992) with that of Thomson (1998) in further view of Elsea (2002) and US 2005/0054092, since as stated above, although desired by Thomson (1998), the teachings of Ratcliff et al. with respect to homologous recombination in mouse ES cells cannot be used without major modifications on human ESCs in order to generate human ESC lines harboring the genetic mutations, even in view of Elsea et al., who merely stated the desired need for human ESCs as a model for genetic diseases, and 2005/0054092, which teaches isolation of cells with FACS.

Thus, it is Applicants' position that due the major differences between mouse and human ESCs, and the lack of guidance in Ratcliff et al., Thomson et al., Elsea (2002) and US 2005/0054092 with respect to what modifications are needed for performing homologous recombination in human ESCs, one of ordinary skills in the art would not have been motivated to apply the technology of producing specific

disruptions in mouse ESCs on human ESCs to thereby generate the human ESC lines carrying a disease-causing mutation in a genomic polynucleotide sequence thereof as claimed.

Withdrawal of the rejection is respectfully requested.

Ratcliff et al., 1992, Thomson et al., 1998, Elsea et al., 2002 and US Pat. No. 5,972,955

The Examiner has rejected claims 57 and 81 under 35 U.S.C. 103(a) as being unpatentable over Ratcliff (Transgenic Res. 1:177-181, 1992) when taken with Thomson et al., (Science, 282:1145-1147, 1998) in further view of Elsea et al., (ILAR Journal, 43:66-79, 2002) as applied to claims 52, 55, 56, 58-60, 74, 75, 78-80, 82 above, and further in view of US Pat. No. 5,972,955. Specifically, the Examiner states that Ratcliff, Thomson and Elsea which are described above do not specifically teach sequences such as those recited in claims 57 and 81, however, US Pat. No. 5,972,955 teaches an exact match of SEQ ID NO:24. Thus, the Examiner states that it would have been obvious for the ordinary skilled artisan to modify the teachings of Ratcliff, Thomson and Elsea to produce human ES cells carrying a mutation such as W1282X as set forth in SEQ ID NO:24, associated with cystic fibrosis, with a reasonable expectation of success. The Examiner's rejection is respectfully traversed.

Applicants point out that a *prima facie* case of obviousness has not been properly set by combining the art of Ratcliff (1992) with that of Thomson (1998) in further view of Elsea (2002) and US Pat. No. 5,972,955, since as stated above, although desired by Thomson (1998), the teachings of Ratcliff et al. with respect to homologous recombination in mouse ES cells cannot be used without major modifications on human ESCs in order to generate human ESC lines harboring the genetic mutations, even in view of Elsea et al., who merely stated the desired need for human ESCs as a model for genetic diseases, and US Pat. No. 5,972,955, which as stated by Examiner, merely discloses a sequence identical to SEQ ID NO:24.

Thus, it is Applicants' position that due the major differences between mouse and human ESCs, and the lack of guidance in Ratcliff et al., Thomson et al., Elsea (2002) and US Pat. No. 5,972,955 with respect to what modifications are needed for performing homologous recombination in human ESCs, one of ordinary skills in the

art would not have been motivated to apply the technology of producing specific disruptions in mouse ESCs on human ESCs to thereby generate the human ESC lines carrying a disease-causing mutation in a genomic polynucleotide sequence thereof as claimed.

Withdrawal of the rejection is respectfully requested.

In view of the above arguments and remarks, Applicants believe they have overcome the 35 U.S.C. § 103 rejections.

In view of the above amendments and remarks it is respectfully submitted that claims 52, 55-60, 74, 75, 78-84 are now in condition for allowance. A prompt notice of allowance is respectfully and earnestly solicited.

Respectfully submitted,

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Date: June 2, 2009

Enclosures:

- Declarations of Inventor Michal AMIT and Joseph ITSKOVITZ-ELDOR under 37 C.F.R. §1.131;
- Declaration of Inventor Michal AMIT under 37 C.F.R. §1.132;
- Declaration of Inventor Joseph ITSKOVITZ-ELDOR under 37 C.F.R. §1.132;
- C.V. and list of publications of inventor Michal AMIT;
- Reference1: Eiges R., et al., 2001, Current Biology, 11:514-518;
- Reference2: Amit et al., 2000, Developmental Biology, 227:271-278
- Reference3: Amit et al. Chapter 7

Establishment of human embryonic stem cell-transfected clones carrying a marker for undifferentiated cells

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Human embryonic stem (ES) cells are pluripotent cell lines that have been derived from the inner cell mass (ICM) of blastocyst stage embryos [1–3]. They are characterized by their ability to be propagated indefinitely in culture as undifferentiated cells with a normal karyotype and can be induced to differentiate in vitro into various cell types [1, 2, 4–6]. Thus, human ES cells promise to serve as an unlimited cell source for transplantation. However, these unique cell lines tend to spontaneously differentiate in culture and therefore are difficult to maintain. Furthermore, colonies may contain several cell types and may be composed of cells other than pluripotent cells [1, 2, 6]. In order to overcome these difficulties and establish lines of cells with an undifferentiated phenotype, we have introduced a reporter gene that is regulated by a promoter of an ES cell-enriched gene into the cells. For the introduction of DNA into human ES cells, we have established a specific transfection protocol that is different from the one used for murine ES cells. Human ES cells were transfected with enhanced green fluorescence protein (EGFP), under the control of murine *Rex1* promoter. The transfected cells show high levels of GFP expression when in an undifferentiated state. As the cells differentiate, this expression is dramatically reduced in monolayer cultures as well as in the primitive endoderm of early stage (simple) embryoid bodies (EBs) and in mature EBs. The undifferentiated cells expressing GFP can be analyzed and sorted by using a Fluorescence Activated Cell Sorter (FACS). Thus, we have established lines of human ES cells in which only undifferentiated cells are fluorescent, and these cells can be followed and selected for in culture. We also propose that the pluripotent nature of the culture is made evident by the ability of the homogeneous cell population to form EBs. The ability to efficiently transfect human ES cells will provide the means to study and manipulate these cells for the purpose of basic and applied research.

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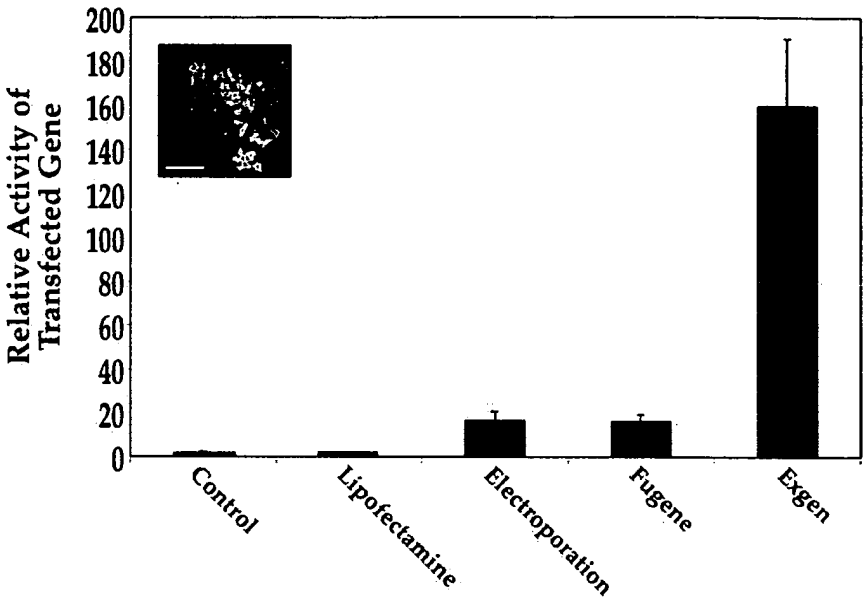
Results and discussion

The objective of this study was to obtain pure clones of human ES cells that are genetically modified so that their undifferentiated phenotype can be followed and selected for in vitro. Thus, we aimed at introducing the EGFP reporter gene under the control of a promoter of an ES cell-enriched gene into human ES cells. By tagging the undifferentiated cells with GFP, we wished to monitor the differentiation status of the cells in culture during growth and propagation as well as following spontaneous and induced differentiation. For this purpose, we chose to use the well-characterized promoter sequence of the murine *Rex1* gene [7]. *Rex1* is a retinoic acid-regulated zinc finger protein that is expressed in preimplantation mouse embryos (including the inner cell mass), trophoblast, and spermatocytes as well as in undifferentiated murine ES cells and some embryonic carcinoma (EC) cell lines [7, 8]. This gene is rapidly downregulated upon differentiation of the embryonic cells. Hence, by introducing *Rex1*-regulated gene markers (*Rex1*-EGFP) into human ES cells, we should be able to express these markers in pluripotent cells, allowing the determination of the differentiation status of these cells in culture.

In order to introduce *Rex1*-EGFP fusion gene into human ES cells, we had to establish a method to transfect the human embryonic cells with DNA. Although ES-like cell lines are now available from a large array of mammalian species (for a review, see Prelle et al. 1999 [9]), there are no published protocols for DNA transfection in any of the species, other than mice. In the mouse, electropora-

Figure 1



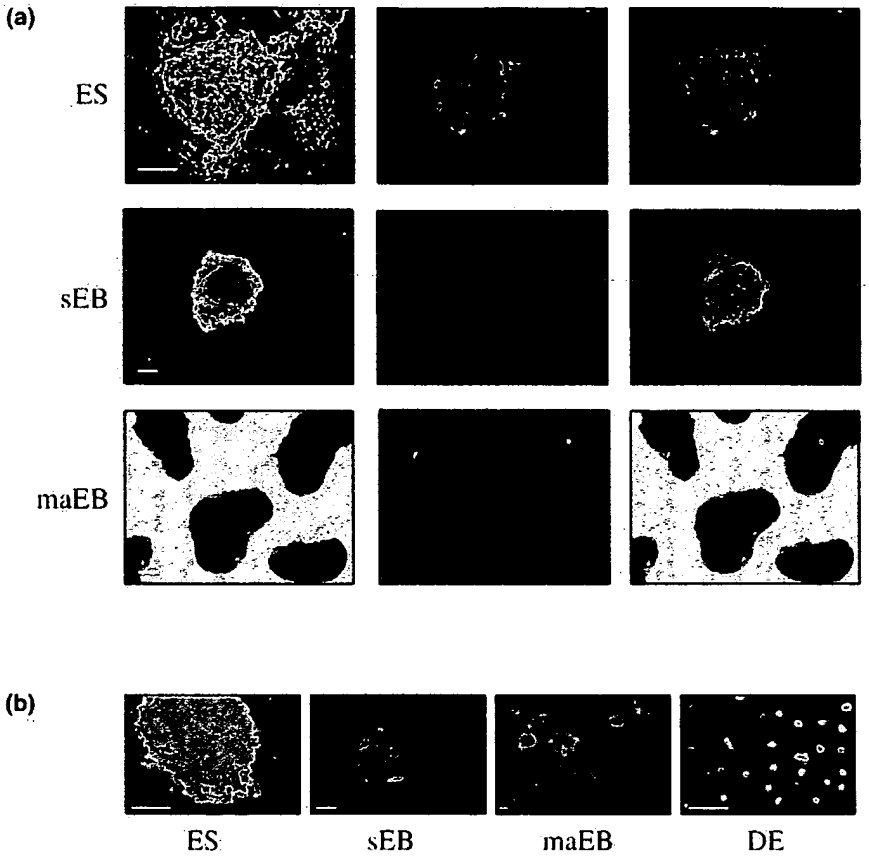
Transfection of DNA into human ES cells. DNA was introduced into human ES cells either by electroporation or by using several commercial reagents such as LipofectAMINE Plus (Life Technologies), FuGENE (Boehringer Mannheim), or ExGen 500 (Fermentas). To determine the efficiency of DNA introduction by each of the methods, the cells were transfected with a construct of firefly Rennila protein under the control of a TK promoter. The cells were harvested 48 hr after transfection, and luminosity of the Rennila protein was monitored using a luminometer. Results are given in the histogram as the relative activity of the transfected gene (luminosity units per mg of total protein), following the subtraction of the values obtained from samples of the appropriate MEF-only controls. Each experiment was repeated three times, and the mean with standard error is shown. Inset: human ES cells transiently transfected with EGFP under the control of the housekeeping gene E1F (elongation factor I) promoter. Note the green fluorescent ES cells that incorporated the foreign DNA. The scale bar indicates 100 μm.

tion was found to be the method of choice for introducing foreign DNA into ES cells [10]. However, human ES cells do not survive electroporation well. Therefore, we compared the efficiencies of several chemical-based methods for the transfection of H9 human ES cells [1] (passage 40–50). Initially, an expression construct of EGFP under the control of the housekeeping gene elongation factor I (E1F) was introduced into human ES cells by several different reagents. Transient expression of the GFP was observed in no more than 10% of the cells, mainly by the human ES cells, and not by the feeder layer of mouse embryonic fibroblasts (MEF) (over 80% of the fluorescent cells had ES cell morphology and resided within the colony boundaries) (Figure 1, inset). To allow quantification and comparison of transfection efficiencies between protocols, a TK-firefly Renilla luciferase reporter gene (Dual Luc Reporter Assay Kit, Promega) was introduced into growing colonies of human ES cells, either by LipofectAMINE Plus (Life Technologies), FuGENE (Boehringer Mannheim), or ExGen 500 (Fermentas) (performed according to the manufacturer's protocols). Cell samples were lysed (using the passive lysis buffer of the assay kit) and evaluated for the efficiency of transient transfection by measuring the relative activity of luciferase in respect to protein concentration (as determined by the Bradford method [BIO-RAD Protein Assay]) 48 hours after transfection. A clear difference between ExGen 500, FuGENE, LipofectAMINE Plus, and electroporation was apparent. Transfection with ExGen 500 seems to deliver DNA into human ES cells in an order of magnitude more efficiently than other reagents that we have examined (Figure 1).

Using the transfection protocol of ExGen 500, a *Rex1*-EGFP expression vector, which includes the neo selectable marker, was delivered into human ES cells. The following day, cells were trypsinized and replated on a feeder of inactivated MEF that was resistant to neomycin (MEF^{Neo+}), allowing the clonal propagation of transfected cells by G418 selection. At 14 days in culture, neomycin-resistant fluorescent colonies were isolated and propagated for several passages while maintaining their level of fluorescence (up to 13 passages), allowing the establishment of individual cell lines. In our experience, stable clones were derived in an efficiency of $\sim 10^{-5}$ of the transfected cells.

Of the various neo resistant colonies, we have established 10 cell lines, 4 of which were examined under different culture conditions (Figure 2). When grown on feeder cells in the presence of leukemia inhibitory factor (LIF) (to support undifferentiated growth), high expression of GFP was detected in the small and densely packed cells of the undifferentiated colony. The fluorescent emission overlaps well with the discrete margins of the colony and is absent in the periphery, where spontaneous differentiation takes place (Figure 2a). When the transfected human ES cells were induced to differentiate by growing as cell aggregates in suspension culture, fluorescence gradually declines, initially, in the outer surface of 4 day old simple

Figure 2

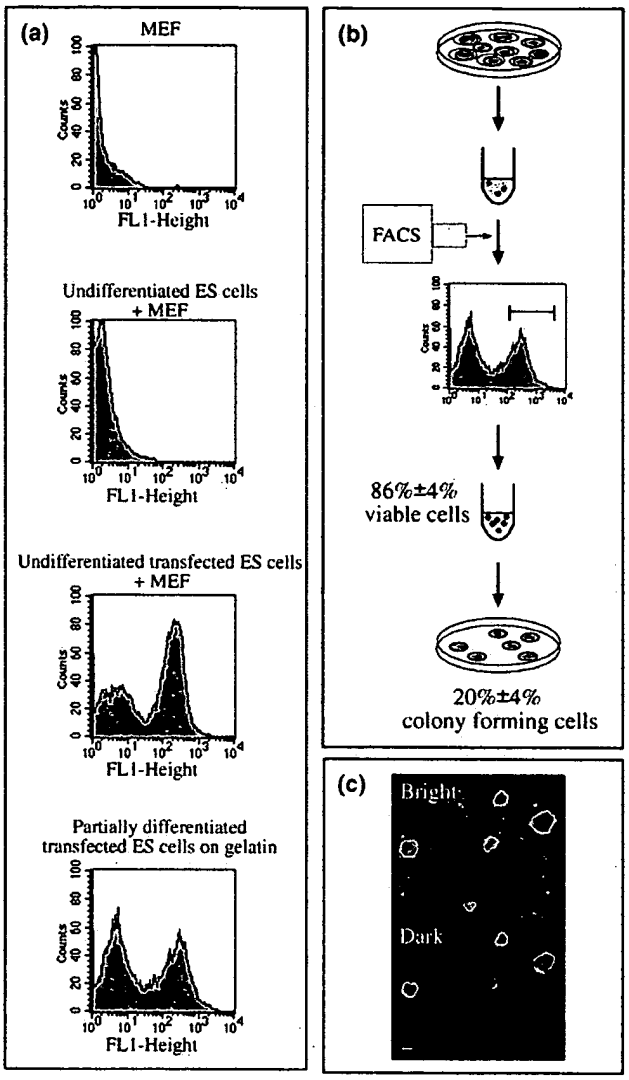


Isolation of human ES clones transfected with a marker for undifferentiated cells. **(a)** Human ES cells underwent stable transfection with EGFP fused to the murine *Rex1* minimal promoter sequence. The transfected ES cells and their differentiated cell derivatives are shown: simple embryoid body (sEB), and mature embryoid bodies (maEBs). The left and middle columns are photos of bright and dark fields, respectively. The right column is the overlay of the two photos. Note that only the undifferentiated cells are fluorescent. The fluorescent ES colony is surrounded by differentiated nonfluorescent cells. The simple EB is labeled only in the middle and not in the peripheral primitive endodermal cells [11]. Mature EBs are generally not fluorescent, and only very distinct areas in them are still fluorescent (probably residual undifferentiated cells). The scale bar indicates 100 μm. **(b)** The stable transfection of human ES cells with a constitutively expressed EGFP construct, driven by the mouse *PGK* promoter. Overlay photos of the dark on bright field of the transfected ES cells (ES) and their differentiated cell derivatives are shown: simple embryoid bodies (sEB), mature embryoid bodies (maEB), and differentiating embryonic cells derived from dissociated embryoid bodies (DE). Note that GFP is expressed by all cells, differentiated and undifferentiated, in the proliferating ES colony as well as by all cells of simple and mature EBs (including those in the outer layer of the sEB, where differentiation of primitive endoderm is taking place in the mouse EBs [11]). The scale bar indicates 100 μm.

EBs, where a layer of primitive endoderm was demonstrated in the mouse [11]. Later, if maintained to form mature EBs (20 days in suspension), the fluorescence practically ceases, apart from a few cores of undifferentiated cells (Figure 2a). This is in contrast to transfections of constructs driven by constitutively expressed promoters (*PGK*, phosphoglycerate kinase 1; and *CMV*, cytomegalovirus), in which expression of GFP was observed in both undifferentiated and differentiated cells of the colony (Figure 2b).

In an attempt to distinguish between populations of undifferentiated and differentiated human ES cells, we have analyzed the *Rex1*-EGFP transfected cell lines by FACS (Figure 3). Cell samples of MEF, undifferentiated human ES cells, and a mixture of undifferentiated and differentiated transfected cell lines were characterized according to their fluorescent emission. As expected, a clear difference in fluorescent intensity exists between the undifferentiated cultures of untransfected and transfected cell lines. In addition, when comparing EGFP-transfected human ES cells to their differentiated derivatives, a reduction in emission intensity is observed (Figure 3a). This shift in fluorescence emission represents a transition, from undifferentiated to differentiated, in the state of the cells. To allow the collection and selective propagation of the most fluorescent cells in the culture, three different GFP-expressing human ES cell lines (3–4 cell sample replicates per clone) were sorted by FACS (Figure 3b). Cell sorting was performed according to the background level of fluorescence that had been obtained by the analysis of untransfected human ES cells. The different cell samples were individually sorted for collection into tubes containing 25,000–50,000 cells each. By comparing the total cell count and the number of viable cells prior and following cell sorting (determined by trypan blue staining), we could show that the FACS procedure had no detrimental effect on cell viability, as 86% of the sorted cells were viable. Moreover, by plating the isolated cells on MEF^{Neo+} 10 cm^2 culture dishes and allowing their propagation in vitro, we demonstrated their ability to develop into undifferentiated fluorescent-labeled human ES colonies, with an efficiency of 20% ± 4% (n = 11) (ranging from 2% to 41%) (Figure 3b). In our procedure, many sorted cells were grown in the same culture dish, potentially allowing

Figure 3



FACS analysis and cell sorting of the transfected human ES cells. **(a)** Human ES cells transfected with *Rex1*-EGFP construct were analyzed by FACS according to the intensity of green fluorescence emission (FL1 height). Cell samples of MEF and undifferentiated human ES cells were used as controls. Fluorescent intensity between undifferentiated human ES cells, transfected human ES cells, and their differentiated cell culture derivatives (obtained by growth on gelatin-coated plates in the absence of LIF and bFGF) was then compared. The high-fluorescent intensity peak represents GFP positive cells, while the low-intensity peak represents background levels that may result either from autofluorescence or residual promoter activity. **(b)** Cell sorting of three GFP-expressing cell lines was performed by FACS. Following trypsin digestion, cell samples (3–4 replicates per clone) were evaluated for percentage of cell viability (84%) and sorted according to the intensity of green fluorescent emission. The collected cell samples (25,000–50,000) were redetermined for cell viability (86%) and replated on MEF^{Neo+} culture dishes (2,500–8,000 cells per dish). Following growth in vitro, cell culture dishes were inspected and recorded for total number of proliferating human ES colonies (20% ± 4% [n = 11]). **(c)** Photos of fluorescent-labeled proliferating human ES colonies (top, bright field; and bottom, dark field) obtained 4 days after cell sorting by FACS.

mutual support of growth and relatively high plating efficiency. This differs from the single cell dilution procedure by which the clonality of human ES cells was conferred [6]. After FACS sorting, the cells have a morphology indistinguishable from that seen before, but we have not yet tested them for pluripotency.

In our research, we have developed a stem cell selection approach in an attempt to facilitate maintenance of human ES cells in vitro. Currently, the available methods applied for this purpose involve the identification and isolation of single colonies under a dissecting microscope; however, these procedures are time consuming and labor intensive. As an alternative, we suggest a method for purifying undifferentiated cells by cell sorting the fluorescent-labeled cells from a mixed population. Similar selection of undifferentiated clones may be achieved by introducing into the cells a gene that enables drug selection, such as neo resistance gene, under the regulation of an ES-specific promoter [12]. By generating pure populations of undifferentiated cells, as described above, we should be able to avoid the loss of human ES cultures due to their spontaneous differentiation in vitro. Our system of introducing a cell-specific selectable marker into the genome of undifferentiated human ES cells provides a model for isolating specific cell types for transplantation from heterogeneous cell cultures obtained by induced differentiation. Similarly, such methods may be considered for eliminating human ES cells by negative selection prior to transplantation of differentiated cells, avoiding the risk of tumor induction.

The expression of *Rex1*-regulated reporter gene by the cells in the growing colony illustrates that these cells maintained their undifferentiated phenotype. In addition, the transfected cells can develop into undifferentiated colonies that maintain their ability to form EBs in vitro. These results support previous work that demonstrated the clonality of human ES cells [6] and the capacity of these homogenous cultures to differentiate into the three germ layers.

Finally, we report the first isolation of genetically engineered human ES cell lines and describe an efficient protocol for transfecting these cells. By introducing genetic modifications into their genome, we should be able to manipulate them in vitro and use them as vectors in cell-based therapies as well as for other biomedical and research purposes.

Materials and methods

Cell culture

Human ES cells (H9 [1], passage 40–50) were cultured on a Mitomycin-C-treated mouse embryonic fibroblast (MEF) feeder layer (obtained from 13.5 day embryos) in 80% KnockOut DMEM medium (GIBCO-BRL), supplemented with 20% KnockOut SR (a serum-free formulation) (GIBCO-BRL), 1 mM glutamine (GIBCO-BRL), 0.1 mM β-mercaptoethanol (Sigma), 1% nonessential amino acids stock (GIBCO-BRL), Penicillin (50 units/ml), Streptomycin (50 μg/ml), and 4 ng/ml basic fibroblast

growth factor (bFGF). The cells were grown in the presence of LIF (10^3 units/ml, GIBCO-BRL), although its necessity for supporting undifferentiated growth in human ES cells is currently unclear [1, 2]. The undifferentiated cell cultures were induced to differentiate in vitro into EBs by omitting LIF and bFGF from the growth media and allowing aggregation in petri dishes [3]. Following the formation of simple EBs by a 5 day cell aggregation step, cell masses were either trypsin dissociated and left to grow as a monolayer on fibronectin-creating cultures of differentiated embryonic (DE) cells [5] or further expanded in suspension and allowed to develop into 20 day old mature EBs (maEBs) (yielding cavitated and cystic EBs). In addition, we allowed some undifferentiated cells to undergo spontaneous differentiation as a monolayer by growing them on 0.1% gelatin-coated plates (Merck) in the absence of LIF and bFGF.

Plasmid construction

Rex1-EGFP and *PGK*-EGFP expression vectors were constructed by the deletion of the *CMV* promoter sequence from pEGFP-N1 (Clontech) and the insertion of either the mouse *Rex1* promoter sequence (700 bp) into the HindIII restriction site or the mouse *PGK* (phosphoglycerate kinase 1) promoter (515 bp) into the EcoRI and BamHI restriction sites. These constructs contained an SV40-driven neo selectable marker. The use of SV40 promoter in our system was sufficient to confer G418 resistance by driving the neo gene, although it was somewhat inefficient in mouse ES cells.

Transfection and establishment of transgenic cell lines

Fully expanded and undifferentiated human ES cells underwent stable transfection with *Rex1*-EGFP, *CMV*-EGFP, or *PGK*-EGFP plasmid DNA by the ExGen 500 transfection system (Fermentas). Transfection of human ES cells was carried out in 6-well trays on MEF, two days after plating, and was performed as described by the manufacturer's protocol. Specifically, 2 μg of plasmid DNA plus 10 μl of the transfecting agent ExGen 500 were added to $\sim 3\times10^5$ cells in a final volume of 1 ml media per well. The cells were centrifuged at 280 × g for 5 min and incubated at 37°C in a moist chamber for an additional 45 min. Residuals of the transfecting agent were removed by washing the cells twice with PBS. The following day, the cells were trypsinized and $\sim 5\times10^5$ were replated on each 10 cm culture dish containing inactivated MEF^{Neo+}. Two days following replating, G418 (200 ng/ml) was administered to the growth medium, allowing the selective propagation of transfected cells in culture. By day 14, neo resistant fluorescent-labeled colonies were identified by a fluorescent microscope (up to 10 colonies per plate). Using our constructs, over 80% of the neo resistant colonies were also GFP positive. Single transgenic colonies were picked by a micropipette, dissociated into small clumps of cells, and transferred into a 24-well culture dish on a fresh feeder of MEF^{Neo+}. The cells continuously proliferated in the presence of G418 and formed a large number of expanding undifferentiated colonies.

FACS analysis and cell sorting

FACS analysis of *Rex1*-EGFP-expressing cells was performed on a FACSCalibur system (Becton-Dickenson), according to their green fluorescent emission. Undifferentiated human ES cells were used to set the background level of fluorescence. Transfected cells, either undifferentiated (grown on MEF cells in the presence of LIF) or partially differentiated (obtained by growth on gelatin in the absence of LIF and bFGF) were analyzed for fluorescence intensity and compared to control cells.

GFP-expressing cell lines were sorted by FACS according to their fluorescence emission. Following trypsin digestion and centrifugation, cell pellets ($\sim 2-5\times10^6$ cells from each cell line) were resuspended in PBS, filtered by a 70 μM cell strainer (Falcon), and divided into four different tubes, which were kept on ice under sterile conditions. Total cell counts and percent of viable cells were determined for each sample by 0.5% trypan blue staining (1:1 volume) prior to analysis and sorting by FACS. Cell samples were sorted for the collection of 25,000–50,000 cells in 50 ml conical tubes (Falcon) precoated with BSA (4% in PBS). Following centrifugation (5 min, 1000 rpm), the cells were resuspended in 0.5 ml PBS, analyzed for cell viability as described above, and plated on MEF^{Neo+} 10 cm culture dishes. Following 4 days in culture in the presence of G418 (200 ng/ml), the cell cultures were inspected under the microscope, and the total number of colonies per plate was recorded.

Acknowledgements

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Clonally Derived Human Embryonic Stem Cell Lines Maintain Pluripotency and Proliferative Potential for Prolonged Periods of Culture

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Embryonic stem (ES) cell lines derived from human blastocysts have the developmental potential to form derivatives of all three embryonic germ layers even after prolonged culture. Here we describe the clonal derivation of two human ES cell lines, H9.1 and H9.2. At the time of the clonal derivation of the H9.1 and H9.2 ES cell lines, the parental ES cell line, H9, had already been continuously cultured for 6 months. After an additional 8 months of culture, H9.1 and H9.2 ES cell lines continued to: (1) actively proliferate, (2) express high levels of telomerase, and (3) retain normal karyotypes. Telomere lengths, while somewhat variable, were maintained between 8 and 12 kb in high-passage H9.1 and H9.2 cells. High-passage H9.1 and H9.2 cells both formed teratomas in SCID-beige mice that included differentiated derivatives of all three embryonic germ layers. These results demonstrate the pluripotency of single human ES cells, the maintenance of pluripotency during an extended period of culture, and the long-term self-renewing properties of cultured human ES cells. The remarkable developmental potential, proliferative capacity, and karyotypic stability of human ES cells distinguish them from adult cells. © 2000 Academic Press

Key Words: human embryonic stem cells; basic fibroblast growth factor; cloning; telomeres.

INTRODUCTION

Human pluripotent cell lines have been derived from preimplantation embryos (embryonic stem cell lines, ES cells; Reubinoff *et al.*, 2000; Thomson *et al.*, 1998) and from fetal germ cells (embryonic germ cell lines, EG cells; Shamblott *et al.*, 1998) that for prolonged periods of culture maintain a stable developmental potential to form advanced derivatives of all three embryonic germ layers. Human ES cell lines have widespread implications for human developmental biology, drug discovery, drug testing, and transplantation medicine. For example, current knowledge of the postimplantation human embryo is largely based on a limited number of static histological sections, and because of ethical considerations, the underlying mechanisms that control the developmental decisions of the early human embryo remain essentially unexplored. Although the mouse is the mainstay of experimental mammalian developmental biology, there are significant differences between early mouse and human development. These differences are especially prominent in the extraembryonic membranes, in the placenta, and in the arrangement of the germ layers at the time of gastrulation. The yolk sac, for example, is a robust, well-vascularized extraembryonic tissue that is important throughout mouse gestation, but in the human embryo, the yolk sac is essentially a vestigial structure during later gestation (Kaufman, 1992; O'Rahilly and Muller, 1987). Human ES cells should provide important new insights into the differentiation and function of tissues that differ significantly between mice and humans.

In addition to advancing basic developmental biology, human ES cells should have practical, applied uses. The differentiated derivatives of human ES cells could be used for: (1) identification of gene targets for new drugs, (2)

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testing the toxicity or teratogenicity of new compounds, and (3) transplantation to replace cell populations destroyed by disease. Potential conditions that might be treated by the transplantation of ES cell-derived cells include Parkinson's disease, cardiac infarcts, juvenile-onset diabetes mellitus, and leukemia (Gearhart, 1998; Rossant and Nagy, 1999). However, the scientific and therapeutic potential of human ES cells critically depends on their long-term proliferative capacity, their developmental potential after prolonged culture, and their karyotypic stability. The originally described human ES and EG cell lines were not clonally derived from single cells and thus pluripotency could be demonstrated only for a population of cells (Reubinoff *et al.*, 2000; Shamblott *et al.*, 1998; Thomson *et al.*, 1998). Therefore, the formal possibility existed that within the population of homogeneous-appearing cells there were actually multiple precursor or stem cells committed to different lineages and that no single cell was capable of forming derivatives of all three embryonic germ layers. The human ES cells we previously derived were isolated and propagated as small clumps because nonhuman primate ES cell colonies dissociated to single cells plate at a very low efficiency (Thomson *et al.*, 1995; Thomson and Marshall, 1998).

Here we describe conditions for clonally deriving human ES cell lines. Two clonally derived human ES cell lines proliferated for a period of at least 8 months after clonal derivation (population doubling (PD) 286 from initial derivation of the parental cell line) and maintained the ability to differentiate to advanced derivatives of all three embryonic germ layers. Both clonal human ES cell lines expressed high levels of telomerase and maintained terminal restriction fragment (TRF) lengths between 8 and 12 kb. In contrast, normal human somatic cells gradually lose telomeric DNA and senesce after 50–80 PD when TRF lengths are about 5–7 kb. Thus, these results clearly demonstrate the pluripotency of single human ES cells and demonstrate the remarkable proliferative capacity of these cells.

MATERIALS AND METHODS

The derivation, routine culture, and characterization of the human ES cell line H9 were previously described (Thomson *et al.*, 1998). Human ES cells were plated on irradiated (35 gray γ irradiation) mouse embryonic fibroblasts. Culture medium for the present work consisted of 80% KnockOut Dulbecco's modified Eagle's medium, an optimized medium for mouse ES cells (Gibco BRL, Rockville, MD), 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, and 1% nonessential amino acids stock (Gibco BRL), supplemented with either 20% fetal bovine serum (HyClone, Logan, UT) or 20% KnockOut SR, a serum replacer optimized for mouse ES cells (Gibco BRL). The components of KnockOut SR have been published elsewhere (Price *et al.*, 1998). In initial cloning experiments, medium was supplemented with either serum or serum replacer and was used either with or without human recombinant basic fibroblast growth factor (bFGF; 4 ng/ml). For prolonged culture, the serum-free medium required supplementation with bFGF.

To determine cloning efficiency, H9 cells were dissociated to single cells for 7 min with 0.05% trypsin/0.25% EDTA, washed by centrifugation, and plated on mitotically inactivated mouse embryonic fibroblasts (10^5 ES cells in triplicate wells of 6-well plates). To confirm growth from single cells for the derivation of clonal ES cell lines, individual cells were selected by direct observation under a stereomicroscope and transferred by micropipette to individual wells of a 96-well plate containing mouse embryonic fibroblast feeders with medium containing 20% serum replacer and 4 ng/ml bFGF. Clones were expanded by routine passage every 7 days with 1 mg/ml collagenase type IV (Gibco BRL).

For karyotype analysis, either standard G banding or multicolor spectral karyotyping (SKY) was performed (Schrock *et al.*, 1996). For SKY analysis, the SKY H-10 kit was used according to the manufacturer's instructions (Applied Spectral Imaging, Inc., Carlsbad, CA). Metaphase figures were dropped onto clean glass slides and treated with combinatorially labeled whole genome painting probes. After stringent washes in 50% formamide, images of metaphase spreads were captured using the Applied Spectral Imaging spectrophotometer and SKY software on a Zeiss Axioplan II microscope. Karyotypes were analyzed and arranged with combined software processing of the image and reverse DAPI banding. For each SKY sample, 5 metaphases were captured and analyzed completely, and 20 metaphases were captured for modal number determination.

Prior to measurement of telomerase activity and telomere length, ES cells expressing TRA-1-60 (a marker of undifferentiated human ES cells) were selected from cultures grown on irradiated mouse embryonic fibroblasts. Cells were dissociated using 0.2% EDTA and then incubated with a monoclonal antibody against TRA-1-60 (gift from Peter Andrews). After washing, cells were incubated with goat anti-mouse IgM-conjugated magnetic microbeads and processed through a MACS magnetic separation column (Miltenyi Biotec, Bergisch Gladbach, Germany) (Walz *et al.*, 1995). In the samples used for these experiments, greater than 90% of the population was positive for TRA-1-60 using flow cytometric analysis. Telomerase activity was measured using the telomeric repeat amplification protocol (TRAP) assay as described (Kim *et al.*, 1994; Weinrich *et al.*, 1997). TRF size was determined using Southern hybridization essentially as described (Allsopp *et al.*, 1992; Harley *et al.*, 1990; Vaziri *et al.*, 1993).

For teratoma formation, H9.1 and H9.2 cells, cultured for 6 months after cloning, were injected into the rear leg muscle of 4-week-old male SCID-beige mice (eight mice total). Cell numbers ranged from 2.5×10^6 cells to 7.5×10^6 cells per injection. Three to four months after injection the mice were sacrificed and the resulting teratomas examined histologically.

RESULTS

To demonstrate the long-term pluripotency and replicative immortality of single human ES cells, we have derived clonal human ES cell lines. These clonal lines have been maintained for over 1 year *in vitro*, and proliferation rate, karyotypes, teratoma formation, telomere length, and telomerase activity have been examined.

The cloning efficiency of human ES cells was extremely poor under previously described culture conditions that included serum. A several-fold increase in cloning efficiency of human ES cells was consistently observed when serum-free medium was used instead of serum-containing

TABLE 1
Cloning Efficiency of H9 Human ES Cells[a]

	(−) bFGF	(+) bFGF (4 ng/ml)
20% Serum	240 ± 28 (0.24)*	260 ± 12 (0.26)*
20% Serum replacer	633 ± 43 (0.63)†	826 ± 61 (0.83)‡

[a] Values are expressed as the mean numbers of colonies resulting from 10^5 individualized ES cells plated ±SE (figures in parentheses represent percentage colony cloning efficiency).

*,†,‡ Means with different superscripts differ significantly ($P < 0.05$, Tukey-Kramer HSD.

medium (Table 1). The addition of bFGF to the medium altered the morphology of human ES cells, resulting in smaller cells in tighter colonies (Fig. 1A). The long-term culture of human ES cells in the presence of serum does not require the addition of exogenous bFGF and the addition of bFGF to serum-containing medium did not significantly increase human ES cell cloning efficiency (Table 1). However, in serum-free medium, bFGF increased the initial cloning efficiency of human ES cells and bFGF was required for continued undifferentiated proliferation. In serum-free medium lacking bFGF, human ES cells became uniformly differentiated by 2 weeks after plating (Fig. 1B).

To avoid the possibility that some of the colonies that grew were not from individual cells, but were from rare, small clumps of cells remaining after dissociation, the H9 cell line was recloned by placing cells individually into wells of a 96-well plate under direct microscopic observation. Of 384 H9 cells (at PD 122) plated into 96-well plates, 2 clones were successfully expanded (H9.1 and H9.2). Both of these clones were subsequently cultured continuously in medium supplemented with serum replacer and bFGF. H9.1 and H9.2 cells maintained a normal XX karyotype even after more than 8 months of continuous culture after clonal derivation (Fig. 2).

Population doubling time was measured in the parent line and in both clonal lines and no significant differences were found between the parent line and the clonal lines. The average population doubling time for 10 separate determinations was 35.3 ± 2.0 h (mean ± standard error of the mean). Because of the considerable cell death observed in these human ES cell cultures, this population doubling time may underestimate the replication rate of the ES cells that survive. In addition, evaluation of the karyotype in the H9 parental population 6 months after derivation (PD 122) presented a normal XX karyotype by standard G-banding techniques (20 chromosomal spreads analyzed). However, 7 months after derivation, in a single karyotype preparation, 4/20 spreads demonstrated random abnormalities; one with a translocation to chromosome 13 short arm, one with an inverted chromosome 20, one with a translocation to the number 4 short arm, and one with multiple fragmentation. Subsequently, at 8, 10, and 12.75 months after derivation (PD 260), H9 cells exhibited normal karyotypes in all 20 chromosomal spreads examined. Both H9.1 and H9.2 also exhibited normal karyotypes 8 months after derivation.

Telomerase activity was high in H9, H9.1, and H9.2 cells at all time points examined (Fig. 3), ranging from about 52 to 196% of that found in H1299, a lung tumor cell line. In these experiments, the human ES cells were separated from fibroblast feeders by magnetic bead sorting to greater than 90% of the cells as determined by Tra-1-60 expression. Even though telomerase activity was also found in the mouse feeder cells (MEF, Fig. 3), the activity in MEF is 4–15% of that found in human ES cells. Therefore, it is unlikely that

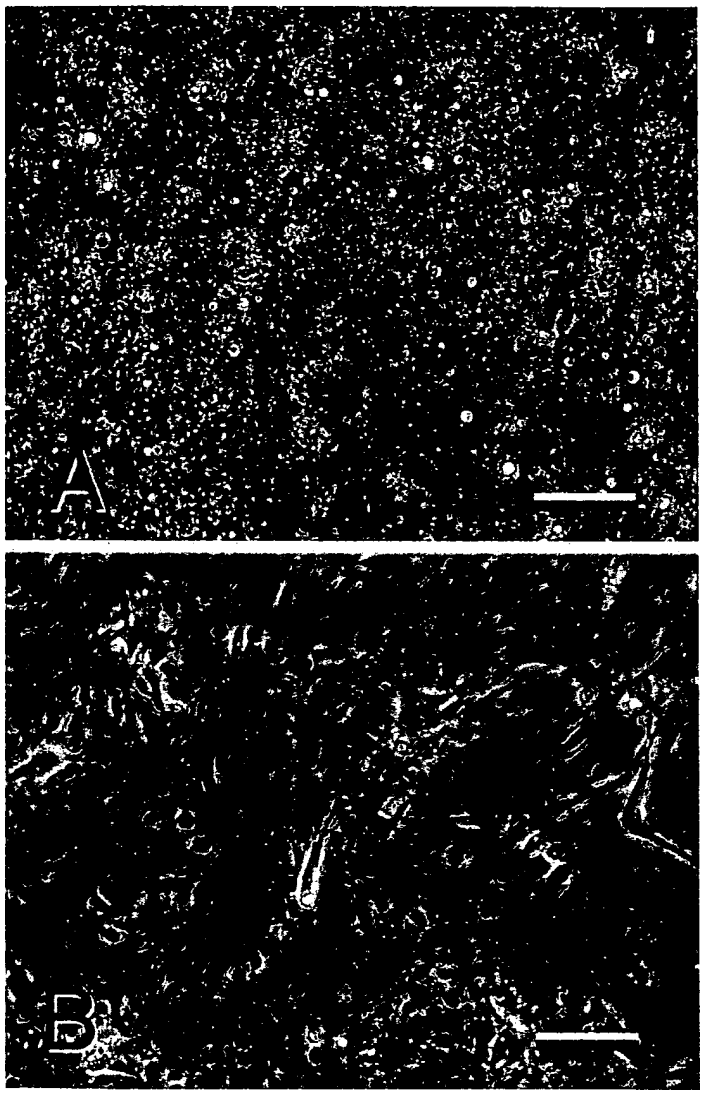

FIG. 1. H9 human ES cells cultured for 14 days in serum-free medium in the presence (4 ng/ml) (A) or absence (B) of bFGF. ES cells plated in serum-free medium in the presence of bFGF continued active, undifferentiated proliferation throughout the culture period. ES cells plated in the absence of bFGF uniformly differentiated into a flattened, epithelial morphology by the end of the 14-day culture period. Bar, 200 μm.

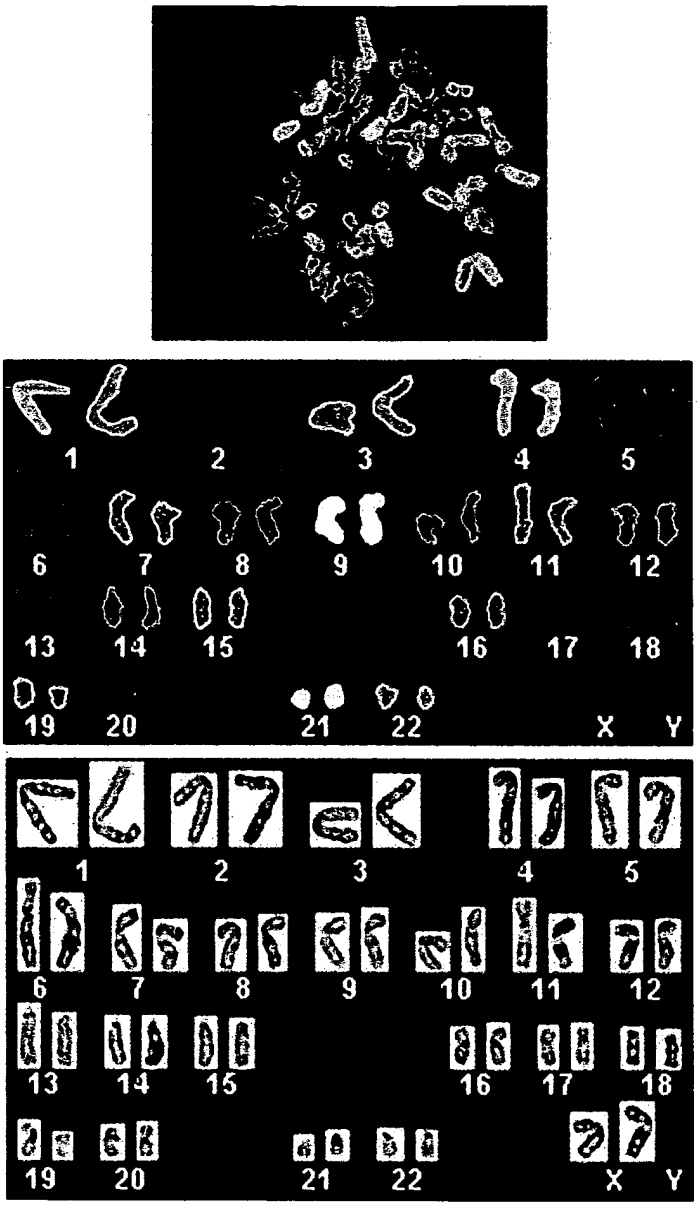

FIG. 2. Metaphase preparation from H9.2 at 8 months (PD 175) of continuous culture. Captured metaphase spectral image (top); classified processed image (middle); reverse DAPI image (bottom). Karyotype: 46, XX normal female.

the telomerase activity seen in the human ES cells is entirely due to the <10% contamination from the feeders.

In general, the presence of telomerase activity is associated with a maintenance of telomere length. H9.1 and H9.2 clones showed roughly stabilized telomere lengths, between 8 and 12 kb. As seen in other populations of telomerase-positive cells, changes in telomere length did not correlate with telomerase activity. At PD 57 after clonal derivation, H9.2 cells had a telomere length of about 8 kb which increased to 13 kb by PD 143. H9.1 cells showed an initial decrease in telomere length followed by an increase to about 11 kb at PD 123. On the other hand, the parental cell line, H9, demonstrated an initial decrease in telomere length from 14 kb at PD 71 (passage 15) to 9 kb at PD 200 (passage 42) (Fig. 3). Cells at later PD will need to be examined to determine if telomeres continue to shorten. However, to date, H9 is still proliferating well at PD 304.

The H9.1 and H9.2 clones maintained the potential to form derivatives of all three embryonic germ layers after long-term culture under serum-free conditions. After 6 months of culture (PD 122), H9.1 and H9.2 clones were confirmed to have normal karyotypes and were then injected into SCID-beige mice. Both H9.1 and H9.2 cells formed teratomas that contained derivatives of all three embryonic germ layers, including gut epithelium (endoderm); embryonic kidney, striated muscle, smooth muscle, bone, and cartilage (mesoderm); and neural tissue (ectoderm) (Fig. 4). The range of differentiation observed within the teratomas of the high-passage H9.1 and H9.2 cells was comparable to that observed in teratomas formed by low-passage parental H9 cells.

DISCUSSION

This report addresses two critical issues: the pluripotentiality and the long-term replicative capacity of clonal human ES cell lines. The previously described human ES cell lines appeared to be a morphologically homogeneous population of cells and expressed cell surface markers characteristic of clonally derived primate ES cells (Reubinoff *et al.*, 2000; Thomson *et al.*, 1998, 1995; Thomson and Marshall, 1998). However, because these human ES cell lines were not clonally derived, pluripotency could be demonstrated only for a population of cells and not for individual ES cells. Our successful cloning of human ES cell lines demonstrates that individual cells are indeed pluripotent and that such individual pluripotent ES cells were present after at least 6 months of continuous culture of the parental H9 cell line. Furthermore, these clonally derived cell lines demonstrated a developmental potential after more than 6 months of additional culture (12 months after the derivation of the parental cell line) that was comparable to low-passage ES cells.

The telomere hypothesis suggests that critical telomere shortening signals cell senescence (Harley *et al.*, 1990; Vaziri *et al.*, 1993) and that telomerase can prevent senescence by maintenance of telomere length (Bodnar *et al.*, 1998; Jiang *et al.*, 1999). This hypothesis is supported by the presence of high telomerase activity in cells with extensive replicative capacity such as germ cells or tumor cells (Chiu and Harley, 1997). The telomerase activity seen in human ES cells, especially in the cell lines which have survived approximately 300 population doublings, i.e., four to six times the life span of normal somatic cells, suggests that these cells are immortal.

Telomere lengths in cloned human ES cells showed some variations from passage to passage, but are stabilized over-

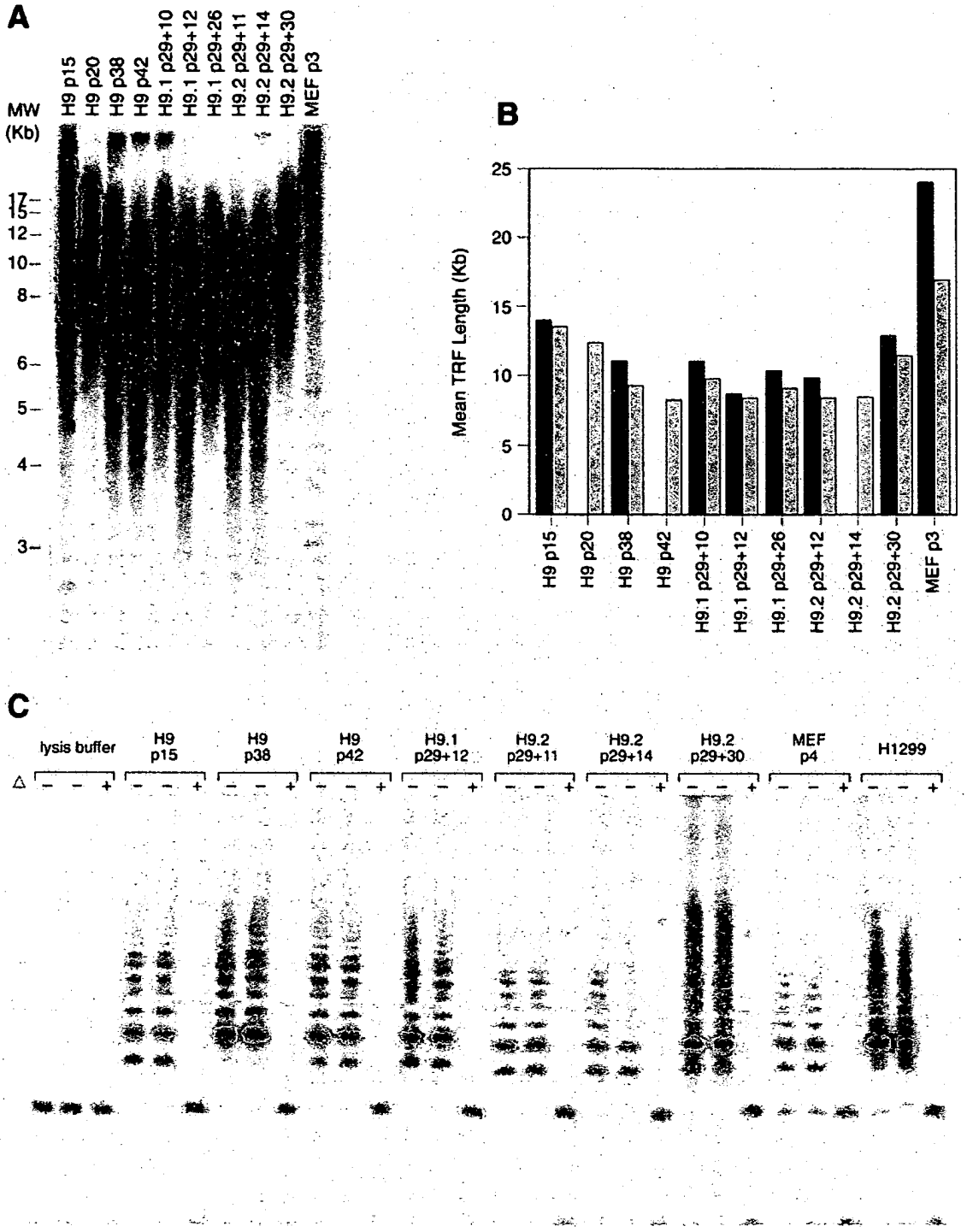

FIG. 3. Telomere length and telomerase activity in human ES cells. (A) Southern analysis of terminal restriction fragment (TRF) from the H9 parent line and two clonal lines, H9.1 and H9.2, at different times *in vitro*. Passage number for the clones is represented as number of passages following subcloning at passage 29 (p29 + *X*). Cells were passaged at 7-day intervals. MEF indicates irradiated mouse primary embryonic fibroblasts. (B) Mean TRF lengths. Telomere length was quantified using a PhosphorImager and ImageQuant software. The mean TRF length for each lane is an integral function based on the densitometric readings in reference to the standards for each gel (A). Mean TRF length was calculated from replicate analyses: light gray bars represent data for gel A, dark gray bars represent replicate analysis of selected samples from gel A. (C) TRAP analysis of telomerase activity in human ES cells. The TRAP assay is a primer extension assay in which telomerase synthesizes telomeric repeats onto oligonucleotide primers. The telomerase extension products serve as a template for PCR amplification. The laddering in the TRAP gel represents increasing numbers of telomeric repeats in which radionucleotides are incorporated. Samples were run in triplicate with the third sample as a negative control in which cell lysates were heat inactivated prior to the assay (indicated by the Δ). The positive control was a cell extract from the telomerase-expressing tumor line H1299 cells.

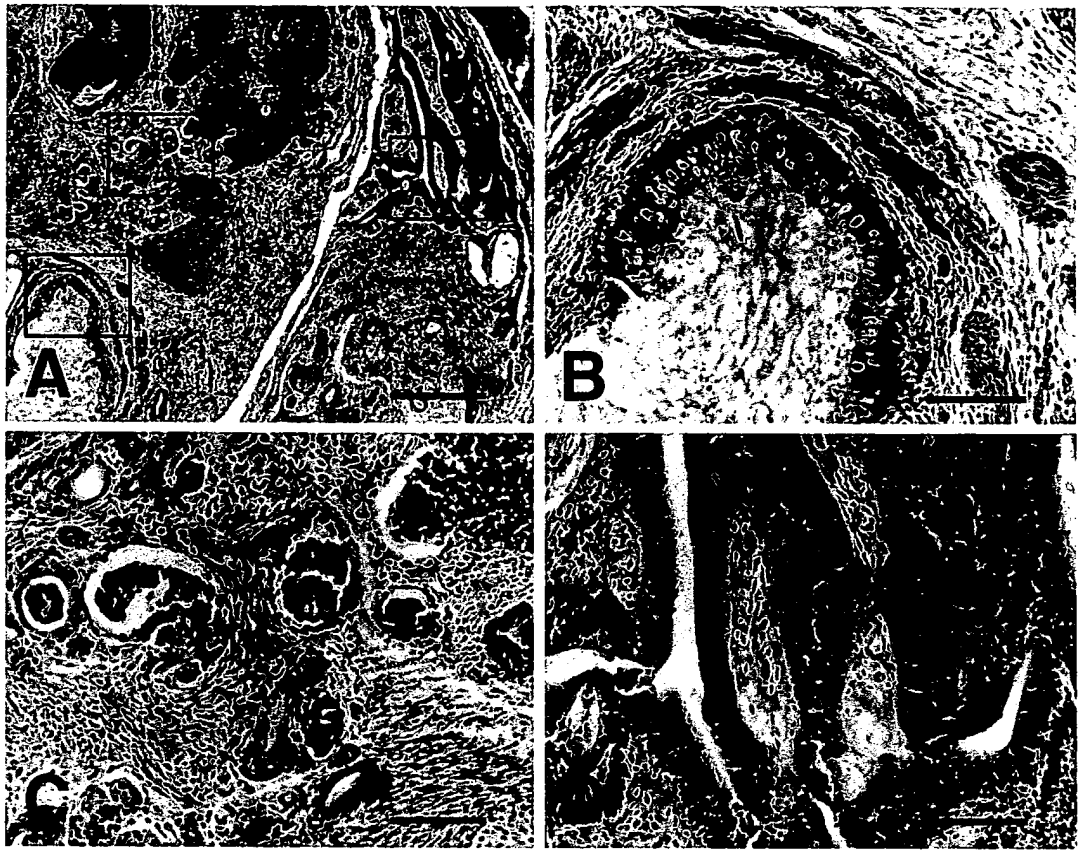

FIG. 4. Teratomas from H9.1 cells. Approximately 5×10^6 H9.1 cells that had been cultured for 6 months (PD 131) after clonal derivation were injected into the hind leg musculature of a SCID-beige mouse. The resulting tumor was harvested and examined 3 months after injection. (A) Low-power view of a field that exhibits differentiated derivatives of all three embryonic germ layers. Areas enclosed in boxes are enlarged in B, C and D (bar, 500 μm). (B) Gut epithelium with adjacent smooth muscle (bar, 100 μm). (C) Embryonic glomeruli and renal tubules (bar, 100 μm). (D) Neural epithelium (bar, 100 μm).

all. This could be due to heterogeneity in the differentiated state of the cultures or could be due to variation or drift in the abundance of factors which establish the equilibrium point for telomere length, in which gain due to telomerase activity matches loss due to incomplete replication and degradation (Chiu *et al.*, 1996; Vaziri *et al.*, 1994). The parental H9 ES cell line showed a decrease in telomere length with increasing population doublings. Despite the telomere shortening, H9 cells are still proliferating well at PD 304. Other immortalized cells also demonstrate telomere shortening in the presence of telomerase and continued proliferation. For example, endothelial cells engineered to express telomerase continue to proliferate but show decreased telomere length which eventually stabilizes (Yang *et al.*, 1999). We are continuing to monitor telomere length in H9 cells.

Serum is a complex mixture that can contain compounds both beneficial and detrimental to human ES cell culture. Different serum batches vary widely in their ability to support vigorous undifferentiated proliferation of human ES cells. Replacing serum with defined components should reduce the variability of experiments associated with serum batch variation and should allow more carefully defined differentiation studies. The consistently lower cloning efficiency in medium containing serum suggests the presence of compounds that are detrimental to stem cell survival when the cells are dispersed to single cells. Although we were able to sustain human ES cells for prolonged periods in a commercially available serum substitute, substantial improvements to the serum-free culture of human ES cells are still needed. Most important, the present culture conditions support a cloning efficiency of human ES cells (<1%) that is considerably lower than the cloning efficiency of mouse ES cells. Unless cloning efficiency is increased significantly, techniques standard to mouse ES cells, such as homologous recombination, will be very difficult to apply to human ES cells.

The fibroblast feeder layer remains the most poorly defined component of the human ES cell culture environment. Unlike undifferentiated mouse ES cells, which can be cultured using leukemia inhibitory factor (LIF) in the absence of fibroblasts (Smith *et al.*, 1988; Williams *et al.*,

1988), under current culture conditions, addition of LIF to the medium does not allow the culture of human ES cells in the absence of feeder layers (Thomson *et al.*, 1998). If LIF and bFGF are added in combination, human ES cells are still lost to differentiation in the absence of fibroblasts (not shown). To date we have also been unable to demonstrate beneficial effects of exogenously added LIF in the presence of fibroblasts (not shown). However, fibroblasts can produce LIF, and given the importance of LIF in the culture of human EG cells (Shamblott *et al.*, 1998), further examination of the role of LIF in human ES cell self-renewal is warranted. Identifying the factors that the fibroblasts produce that promote human ES cell renewal will be critical to the large-scale growth of ES cells, because the feeder layers are labor intensive to prepare and because variation between batches of fibroblasts can introduce undesirable variation and complexity to experiments. It is unknown, for example, whether the beneficial effects of bFGF on undifferentiated human ES cell growth are mediated through the fibroblasts, the ES cells, or both.

Because current culture conditions are suboptimal, a significant percentage of the ES cells die at each passage, even when they are passaged in clumps. For this reason, there is selective pressure for any rare cell population that has an enhanced survival at splitting or an increased rate of proliferation. Improved passaging and culture conditions that increase cloning efficiency should reduce this selective pressure. Given this selective pressure, the karyotypic stability of human ES cells is remarkable, but clones with abnormal karyotypes and a selective growth advantage should eventually take over a culture. Therefore, it is likely that it will be necessary to periodically subclone human ES cells to maintain a euploid population. In a single sample of H9 cells at 7 months of culture, we observed a subpopulation (4 of 20 chromosomal spreads) with abnormal karyotypes. These were the only abnormal karyotypes we observed, and curiously, subsequent karyotypes of the uncloned H9 cells at 8, 10, and 12.75 months of culture revealed 20 of 20 normal chromosomal spreads. We have yet to observe abnormal karyotypes in the H9.1 or H9.2 cells, even at 8 months after their clonal derivation. Thus, although the useful long-term expansion of human ES cells could be somewhat limited by a need to subclone periodically, our results suggest that this need will be infrequent. Ultimately, the slow accumulation of genetic mutations may place an upper limit on the useful long-term culture of human ES cells. Although these considerations may place some practical constraints on the maintenance of human ES cells, the potential uses of ES cells as biological research tools and as therapeutic agents remain unparalleled.

ACKNOWLEDGMENTS

We thank Peter Andrews for the gift of the TRA-1-60 antibody; Yoko Oei and Kathy Ng for technical assistance; and Jane Lebkowski, Calvin Harley, and Ruth Singer for critically reading the manuscript. This work was supported by the Geron Corporation (Grant 133-BU18) and the fund for Medical Research and Development of Infrastructure and Health Services, Rambam Medical Center.

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Subcloning and Alternative Methods for the Derivation and Culture of Human Embryonic Stem Cells

Michal Amit, Hanna Segev, Dorit Manor, and Joseph Itskovitz-Eldor

1. INTRODUCTION

Human embryonic stem (ES) cell lines may have broad applications, including the study of development and the differentiation process, lineage commitment, self-maintenance, and precursor cell maturation. They may also serve as models in research done on the functions of genes and proteins, drug testing, and drug toxicity. The first human ES cells were derived by Thomson and colleagues *(1)* from the inner cell mass (ICM) of surplus blastocysts donated by couples undergoing in vitro fertilization treatments. These lines met most of the criteria for ES cell lines listed in Table 1, but their clonality was not tested in that study. Also, the ability of human ES cells to contribute to embryonic development in chimeric embryos cannot be examined for obvious ethical reasons. Since the first report on human ES cell derivation, several other groups have reported the derivation of additional lines *(2–4)*. At present, there are more than 70 human ES cell lines in several laboratories around the world, according to a list published by the National Institutes of Health (NIH; www.nih.gov/news/stemcell/index). Although the NIH list does not offer full information on all the lines fulfilling all the ES cell criteria listed in Table 1, it suggests that the derivation of human ES cells is a reproducible procedure with reasonable success rates.

This chapter focuses on alternative methods for the derivation and maintenance of human ES cell lines, the derivation of genetically compromised human ES cell lines, and the subcloning of human ES cells parental lines.

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Table 1
Main Features of ES Cells

1. Derived from the preimplantation embryo
2. Pluripotent, capable of differentiating into representative cells from all embryonic germ layers
3. Immortal, with prolonged proliferation at the undifferentiated stage (self-maintenance), and expression of high telomerase activities
4. Maintaining normal karyotype after prolonged culture
5. Ability to contribute to all three embryonic germ layers, including the germ line, following injection into blastocysts
6. Expressing unique markers like transcription factor *Oct-4* or cell surface markers like SSEA-3, SSEA-4, TRA-160, and TRA-181
7. Clonogenic (i.e., each individual cell possessing the above characteristics)

2. DERIVATION OF THE I SERIES

Embryonic stem cell lines are usually derived by immunosurgery, during which the trophoblast layer of the blastocyst is selectively removed and the intact ICM is further cultured on mitotically inactivated mouse embryonic fibroblasts (MEFs). This process is illustrated in Fig. 1. In our laboratory two human ES cell lines, I-3 and I-6, were derived using rabbit anti-human whole antiserum, and a further pluripotent line, I-4, was derived by the mechanical dissection and removal of the trophoblast layer with 27G needles. In our experience, human ES cell lines may also be derived without the complete removal of the trophoblast. In some cases, when the trophoblast is only partially removed, the ICM continues to grow with the remaining surrounding trophoblast as a monolayer. When the ICM reaches sufficient size, it is selectively removed and propagated. The ES cell lines developed in our laboratory have been grown continuously for over 1 yr (more than 60 passages); I-6 is still continuously growing and has reached 130 passages. Lines I-6 and I-3 were found to express high levels of telomerase activity after being continuously cultured for more than 6 mo. After more than 20 passages of continuous culture, karyotype analysis revealed that I-4 and I-3 are normal XX lines and I-6 has a normal XY karyotype. As previously reported on other human ES cell lines *(1,2)*, these cells strongly expressed surface markers that are typical of primate ES cells: stage-specific embryonic antigen-4 (SSEA-4), tumor rejection antibody (TRA)-1-60, and TRA-1-81, with weakly positive staining for SSEA-3 and negative staining for SSEA-1. The overall success rate of derivation was 60%, which is consistent with other reports on human ES cell line derivation *(1,2)*. Table 2 summarizes the characteristics of the new human ES cell lines.

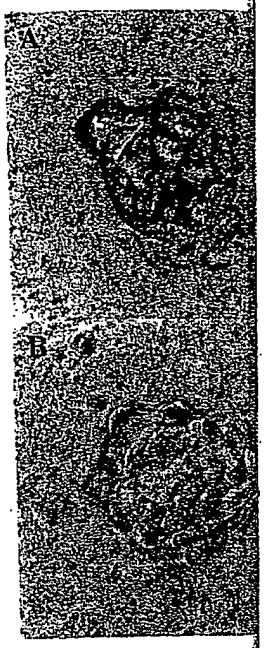

Fig. 1. Immunosurgery of
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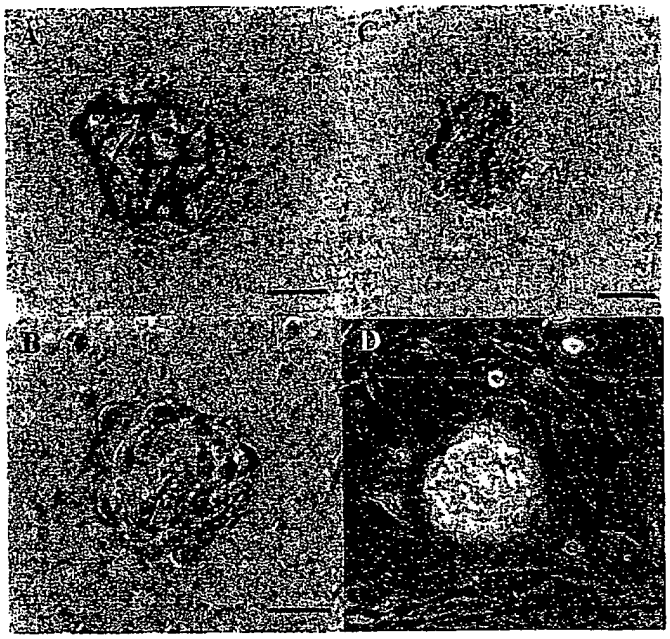

Fig. 1. Immunosurgery of a human blastocyst. **(A)** Donated human embryo produced by in vitro fertilization, post-PGD, which continued to develop to the blastocyst stage. **(B)** Human blastocyst after zona pellucida removal by Tyrode's solution, during exposure to rabbit anti-human whole antiserum. **(C)** The same embryo after exposure to guinea pig complement. **(D)** The intact ICM immediately after immunosurgery on mitotically inactivated MEF feeder layer. Bar = 50 µm. (Reprinted with permission from ref. *4*.)

Following injection into severe combined immunodeficient (SCID)-beige mice, the I-3 and I-6 lines created teratomas that contained representative tissues from all three embryonic germ layers. The I-4 line has not yet been examined. All three lines formed embryoid bodies (EBs) after their removal from the feeder layer or when grown in crowded cultures for more than 3 wk. Thus, these cells answer the criteria for human ES cells.

3. ALTERNATIVE METHOD FOR THE DERIVATION OF PLURIPOTENT HUMAN ES CELL LINES: THE J SERIES

As mentioned earlier, ES cell lines are traditionally derived by either immunosurgery or the mechanical separation of the ICM from the embryo

Table 2
Characterization of Human ES Cell Lines I and J

	I-3	I-4	I-6	J-3	J-6
Year of derivation	7/2000	7/2000	7/2000	1/2001	7/2002
Embryos[a]	Frozen, blastocyst	Frozen, blastocyst	Frozen, blastocyst	Fresh, morula	Fresh, blastocyst
Karyotype	Normal XX	Normal XX	Normal XY	Normal XY	Normal XX
Formation of teratomas	+	To be done	+	+	To be done
EB formation	+	+	+	+	+
TRA-1-60	+++	To be done	+++	To be done	To be done
RRA-1-81	+++	To be done	+++	To be done	To be done
SSEA-1	–	To be done	–	To be done	To be done
SSEA-3	+	To be done	+	To be done	To be done
SSEA-4	+++	To be done	+++	To be done	To be done
Continuous culture	Over 112 passages	Over 60 passages	130 passages and still going	Over 116 passages	Over 54 passages

[a] All embryos were donated by couples undergoing an in vitro fertilization treatment at Rambam Medical Center. The couples voluntarily sign a consent form, which complies with the NIH guidelines.

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at the blastocyst stage. Soon after implantation, the ICM develops into a layer of primitive endoderm, which gives rise to the extraembryonic endoderm and a layer of primitive ectoderm, which gives rise to the embryo proper and to some extraembryonic derivatives *(5)*. After implantation and gastrulation, cells become progressively restricted to a specific lineage. It should be noted, however, that these pluripotent ES cells proliferate and replicate in the intact embryo only for a limited period of time before they commit to specific lineages. The pluripotency of human postimplantation embryonic cells, between the time of implantation and the gastrulation process, has never before been examined.

In order to explore the pluripotency of postimplantation embryonic cells, we used an alternative method to derive pluripotent cell lines. In this method, the trophoectoderm layer is not removed. The trophoblast-surrounded ICM is plated on MEFs. In some cases, the embryo continues to develop as a complete embryo and creates a small cyst. Several days later, the cyst and surrounding trophoblast cells are removed, and the pluripotent-resembling cells are further cultured and propagated. The resulting cell line, J-3, exhibits the main features of human ES cell lines: It grows at the undifferentiated stage for over a year, expresses high levels of telomerase activity, creates EBs when grown in suspension, sustains normal XY karyotype, expresses surface markers typical of human ES cells, and differentiates into the three germ layers following injection into SCID-beige mice. Thus, although the J-3 cell line was derived from an embryo in the "postimplantation" stage, it remained pluripotent and immortal. An additional line, J-6, was derived using the same method. Although this line requires further characterization, the method of deriving these unique postimplantationlike cell lines seems to be reproducible. The features of lines J-3 and J-6 are listed in Table 2.

One of the possibilities is that the J-3 line represents a primitive ectoderm cell population. Rathjen and colleagues demonstrated a method for homogenous differentiation of mouse ES cells into early primitive ectodermlike (EPL) cells using conditioned medium of Hep G2 cells *(6)*. J-3 cells organize as a columnar epithelium when grown on MEFs as undifferentiated cells, whereas the human ES cells organized as pails of cells at the same culture conditions. J-3 cell nuclei are smaller; therefore, their nucleus to cytoplasm ratio is reduced compared with that of a human ES cell.

Another characteristic of the EPL cells is their tendency to differentiate into nascent mesodermal tissue sooner and at higher levels compared to mouse ES cell under the same differentiation culture conditions *(7)*. In teratomas formed from J-3 cells, we could see a variety of mesodermal tissues, such as cartilage tissues, bone tissues differentiated directly from mesen-

RRA-1-81	+++	To be done	+++	To be done	To be done
SSEA-1	-	To be done	-	To be done	To be done
SSEA-3	+	To be done	+	To be done	To be done
SSEA-4	+++	To be done	+++	To be done	To be done
Continuous culture	Over 112 passages	Over 60 passages	130 passages and still going	Over 116 passages	Over 54 passages

[a]All embryos were donated by couples undergoing an in vitro fertilization treatment at Rambam Medical Center. The couples voluntarily sign a consent form, which complies with the NIH guidelines.

chymal tissues, and smooth or stratified muscle tissues, all in a higher rate than in ES cell-line-derived teratomas.

4. DERIVATION OF HUMAN ES CELL LINES HARBORING SPECIFIC GENETIC DEFECTS

Another possible source of donated embryos for human ES cell derivation is embryos from the preimplantation genetic diagnosis (PGD) program. PGD allows couples who are carriers of genetic diseases to examine the embryos before implantation and to retrieve the healthy embryos only. When the in vitro fertilized embryo reaches the six to eight cell stage, one cell is removed and analyzed for the existence of genetic defects either by polymerase chain reaction (PCR) or by fluorescence *in situ* hybridization (FISH). PGD-donated embryos will allow us to create human ES cell lines that harbor different genetic defects and follow the expression of these diseases during differentiation. Such a process may contribute to the development of drugs or gene therapy designed to treat these genetic diseases.

In our experience, embryos donated from couples undergoing PGD continue to develop in vitro to the blastocyst stage. The zona pellucida (ZP) of these embryos remains fractured after the cell to be analyzed is removed by biopsy. In some cases, during culture to the blastocyst stage, parts of the trophoblast cells seep through the fracture in the ZP. These cells can interrupt the removal of the ZP using pronase or Tyrode's solution, but this could be overcome by the mechanical removal of the ZP. An example of a post-PGD embryo that continued to develop to the blastocyst stage is illustrated in Fig. 1A.

Line J-3, described earlier, was obtained from a surplus embryo that underwent PGD for cystic fibrosis. This cell line was found to be heterozygous for the W1282X mutation—the most common type of cystic fibrosis-causing mutation among Ashkenazy Jews *(8)*. Like other human ES cell lines, the J-3 line meets all of the ES cell criteria listed in Table 1. Two additional ES cell lines were derived from embryos, which underwent PGD. The first line was heterozygous for the Gorlin mutation and the second line was heterozygous for the metachromatic leukodystrophy disease. These lines are still at an early passage and need to be fully characterized for ES cell features.

Gene therapy is often based on targeted correction, using small fragments of a corrected region for the gene. The availability of a line heterozygous of the W1282X cystic fibrosis mutation enables us to develop a targeted correction model for this common mutation *(9)*. The availability of human pluripotent cell lines carrying a mutation of cystic fibrosis may offer a suitable

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5. DERIVATION OF HUM

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5.1. Karyotype Stability

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system for investigating the nature of this disease and its progress. Such an understanding may help in the development of both drug and gene therapy models for cystic fibrosis and other genetic diseases.

5. DERIVATION OF HUMAN ES CELL SUBCLONES

As discussed earlier in this chapter, ES cell lines are derived from the clump of cells in the ICM, which may not represent a homogenous cell population. The criteria for pluripotency usually include the derivation of the stem cell line from a single cloned cell. This eliminates the possibility that several distinct committed multipotential cell types are present in the culture and that, together, they account for the variety of differentiated derivatives produced.

In addition to proving the pluripotency of single human ES cells, single-cell clones may have further applications. They form homogeneous cell populations, which may be instrumental in the development of research models based on gene knockout or targeted recombination. The transfected or knockout cells could be cloned and analyzed individually; clones that express the desired genotype could be further cultured and used for research.

5.1. Karyotype Stability

Any future use of human ES cells for scientific or therapeutic purposes will depend on their ability to proliferate for long periods as undifferentiated cells, without losing their developmental potential or karyotypic stability. All of the reports on human ES cell line derivation specifically state that their karyotypes remain normal after prolonged proliferation *(1,2)*. Two of the human ES cell lines, I-3 and I-6, were tested after 105 and 89 passages of prolonged culture, respectively; Each of the examined cells demonstrated normal karyotype. There are two reports on the karyotype instability of human ES cell parental lines *(10,11)*. Amit et al. *(10)* examined the karyotype of the H-9 parental line after 7, 8, 10, and 13 mo of continuous culture. In only one case, after 7 mo of continuous culture, 4 out of the 20 cells examined demonstrated abnormal karyotypes. Eiges and colleagues *(11)* reported on two cells with trisomy in a stably transfected clone. It is possible that a subpopulation with abnormal karyotype will acquire a selective growth advantage and take over the culture. Therefore, the periodical cloning of cultured human ES cells may be needed. According to the data presented in this section on karyotype stability, the need for cloning in order to maintain a euploid population of human ES cells will be infrequent and will have no influence on the long-term expansion of human ES cells in culture.

In our experience, another advantage of single-cell clones is that they are easier to grow and manipulate in comparison to the parental lines.

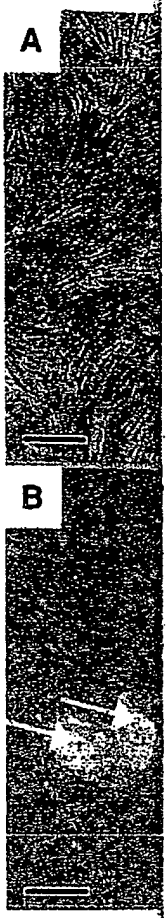
5.2. Methods

Several culture media were tested in order to clone the parental human ES cell lines; medium supplemented with either FBS or serum replacement and either with or without human recombinant basic fibroblast growth factor (bFGF) *(10)*. The serum-free growth conditions supplemented with bFGF have been found to be the suitable ones for the clonal derivation of human ES single-cell lines. The resulting colonies of ES cells in the different cloning conditions are illustrated in Fig. 2. Addition of leukemia inhibitory factor (LIF) or forskolin has no beneficial effect on the cloning rates.

In order to derive the first single-cell clones of human ES cells, H-9 cells were trypsinized to single cells. Each individual cell was plated in a separate well in 96-well plates and grown in serum-free growth conditions. After approx 2 wk of growth, the resulting colonies were passaged and propagated. Consequently, two clonally derived human ES cell lines, H-9.1 and H-9.2, were derived *(10)*. These two single-cell clones proliferated continuously for a period of at least 8 mo after the clonal derivation, maintained a stable and normal karyotype, differentiate in vivo in teratomas into advanced derivatives of all three embryonic germ layers, and expressed high levels of telomerase activity.

To date, eight single-cell clones from five different parental ES cell lines (H-1, H-9, H-13 *[1]*, I-3 and I-6 *[4]*) and one clone from the J-3 pluripotent line have been derived in our laboratory. A summary of all existing single-cell clones is presented in Table 3. Most parental lines have the same cloning efficiency (0.5%). One line, H-1, has a lower cloning efficiency of 0.16% and we were not able to clone line I-4. In fact, all attempts to grow the I-4 line in the serum-free culture conditions failed. During the first passage in the serum-free culture conditions, almost 80% of the cells died. In any passaging technique, the remaining cells did not survive a second passage. However, in a serum-containing culture condition, the I-4 grows with normal survival rates for over 60 sequential passages. The variability between the cell lines with respect to the mechanism underlining the biological versatility of the parental lines and their subclones in their ability for self-renewal and differentiation remains to be determined.

All nine single-cell clones fulfilled the main criteria described for human ES cell lines (Table 1). The clones' karyotype remained normal after more than 6 mo of prolonged culture. All clones formed EBs in suspension, and following injection into SCID mice, they created tertomas containing representative tissues of the three embryonic germ layers (examples are illustrated in Fig. 3).



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the clonal derivation, maintained a
e in vivo in teratomas into advanced
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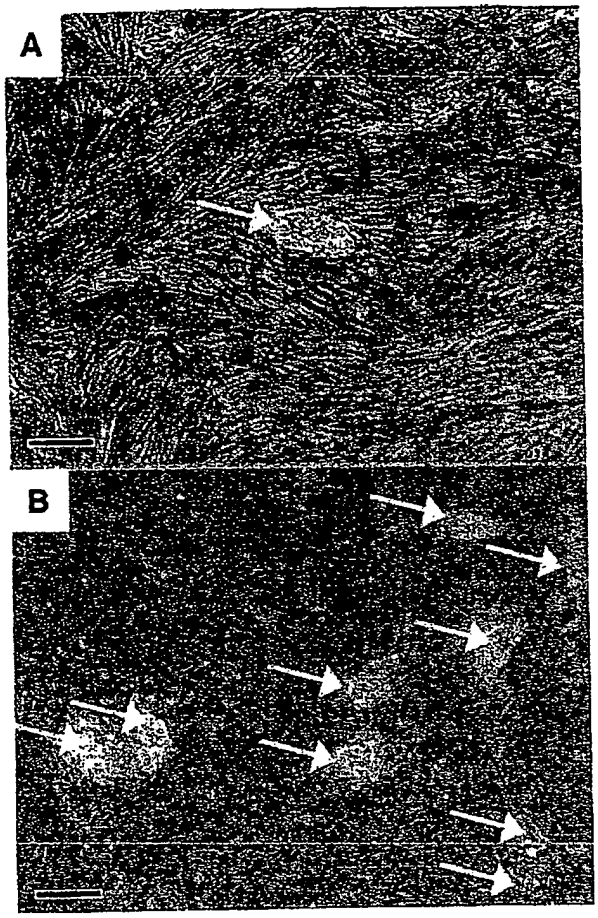

Fig. 2. Resulting colonies of H-9 ES cells in different cloning conditions. (**A**) Resulting colony cloned using medium supplemented with FBS; (**B**) Resulting colonies cloned using medium supplemented with serum replacement and bFGF. Bar: 10 μm.

Preliminary results indicate that there may be a difference between various single-cell clones and the tendency of parental lines to differentiate in a specific direction. In the culture conditions described by Kehat et al., 8% of the EBs formed from H-9.2 single-cell clone beats *(12)*. In the same culture condition, only less than 1% of the EBs formed by H-9 parental line beats (unpublished data). A recent publication by Xu and colleagues *(13)* reported

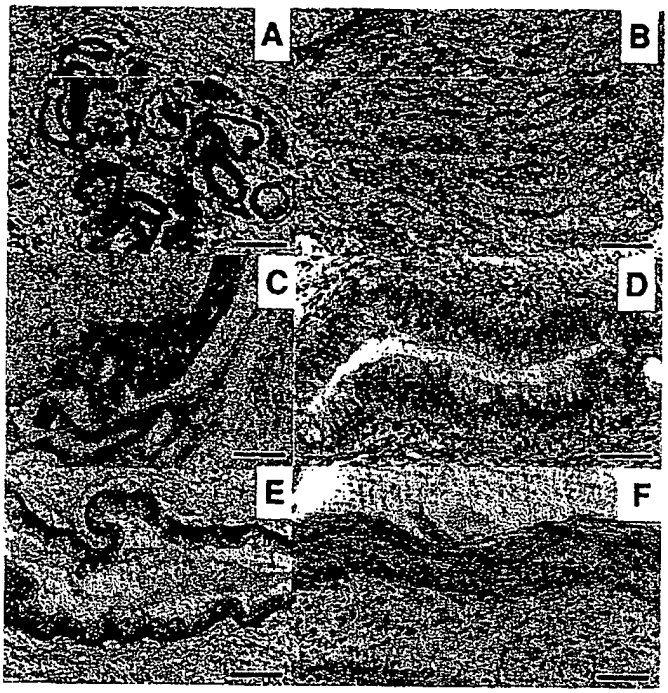

Fig. 3. Teratomas formed by human ES cell single-cell clones in SCID-beige mice. **(A)** Tubules interspersed with structures resembling fetal glomeruli, clone H-9.2; **(B)** embryonal myotubes, clone H-13.1; **(C)** cells producing melanin, clone H-1.1; **(D)** respiratory epithelium, clone H-1.1; **(E)** Mucus-secreting surface epithelium resembling the stratified epithelium found in the stomach, clone H-13.2; **(F)** skinlike epithelium facing a lumen, clone H-9.2 H&E stain. Bar: 50 μm.

on additional culture conditions, including supplement 5-azacytidine, which is suitable for the differentiation of human ES cells into cardiomyocytes. In this differentiation system, H-9.2 clone creates the highest percentage of beating EBs (70%) compared to the parental line H-9 and additional lines (H-1 and H-7).

6. CULTURE OF HUMAN ES CELL LINES IN ANIMAL-FREE CONDITIONS

Human ES cells may also be directly applied in cell-based therapies. In order to clinically use human ES cells, they should correspond with the Food and Drug Administration (FDA) guidelines. One of the major concerns

Table 3
Main Characteristics of the Existing Single-Cell Clones

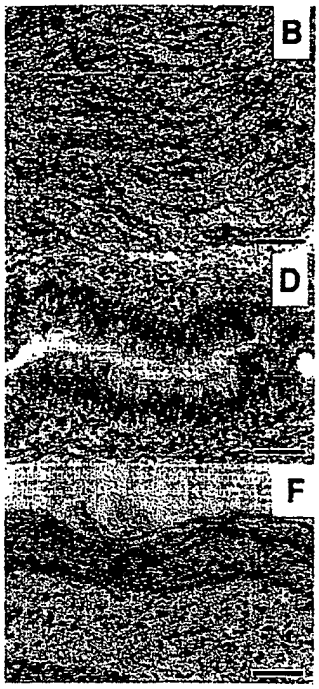

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Table 3
Main Characteristics of the Existing Single-Cell Clones

	Karyotype	EB formation	Formation of teratomas	Continuous culture (mo)	Cloning efficiency of the parental line
H-1.1	Normal XY	+	+	6	1/600
H-9.1	Normal XX	+	+	8	1/400
H-9.2	Normal XX	+	+	8	1/400
H-13.1	Normal XY	+	+	6	1/400
H-13.2	Normal XY	+	+	6	1/400
I-3.2	Normal XX	+	+	6	1/400
I-3.3	Normal XX	+	+	6	1/400
I-6.2	Normal XY	+	+	6	1/400
J-3.2	Normal XY	+	+	10	1/400

related to the application of human ES cells in cell replacement therapy will be the exposure of these cells to retroviruses or other pathogens. These are potentially present in the mouse feeder layer or fetal bovine serum (FBS) with which these cells are derived and grown. Overcoming this problem requires that the human ES cells be derived and cultured in an entirely animal-free environment.

Richards and colleagues offered an animal-free system for the growth and formation of human ES cell lines *(14)*. In their culture system, human ES cells can be grown using a coculture with aborted human fetal-derived feeder layers or human adult fallopian tube epithelial feeder layers. Supplement of 20% human serum replaced the FBS commonly used in the culture of human ES cells. These culture conditions were found to be suitable for human ES cell line derivation. The line derived on a human fallopian tube feeder shares a similar morphology with other human ES cells and expresses *Oct 4* and other ES-cell-specific markers, thus fulfilling the criteria of ES cells.

Exploring the area of animal-free culture systems for human ES cells, we have demonstrated that human foreskin feeder layers support the growth of human ES cells in serum-free conditions. After more than 60 passages (more than 200 doublings), the three human ES cell lines grown on the foreskin feeders (I-3, I-6, and H-9) exhibit all human ES cell characteristics, including teratomas formation, EB creations, expression of typical surface markers, and normal kryotypes. The morphology of a human ES cell colony growing on foreskin is illustrated in Fig. 4A. Nine different foreskin lines that were derived and tested succeeded in supporting a prolonged and undifferentiated proliferation of human ES cells. The cells were grown under serum-free conditions using serum replacement and basic fibroblast growth factor, known to support human ES cell growth *(10a)*.

Human foreskin has several advantages. Unlike aborted fetal fibroblasts, which can grow to reach a certain limited passage, human foreskin lines can grow to reach passage 42. In our experience, high-passage human foreskin feeders can support the growth of human ES cells and can still be frozen and thawed at high efficiency. These feeders may therefore have an advantage when large-scale growth of human ES cells is concerned. Furthermore, the donation of foreskin from circumcised babies has no ethical implications such as those accompanying the donation of aborted fetus. The ability of foreskin feeders to support the *derivation* of human ES cell lines has yet to be determined.

The ideal solution to an animal-free method for growing human ES cells will be the ability to grow these cells on a matrix using serum-free medium.

Fig. 4. Human ES cells c
(A) Human ES cell colony fro
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I-6 line after several passages
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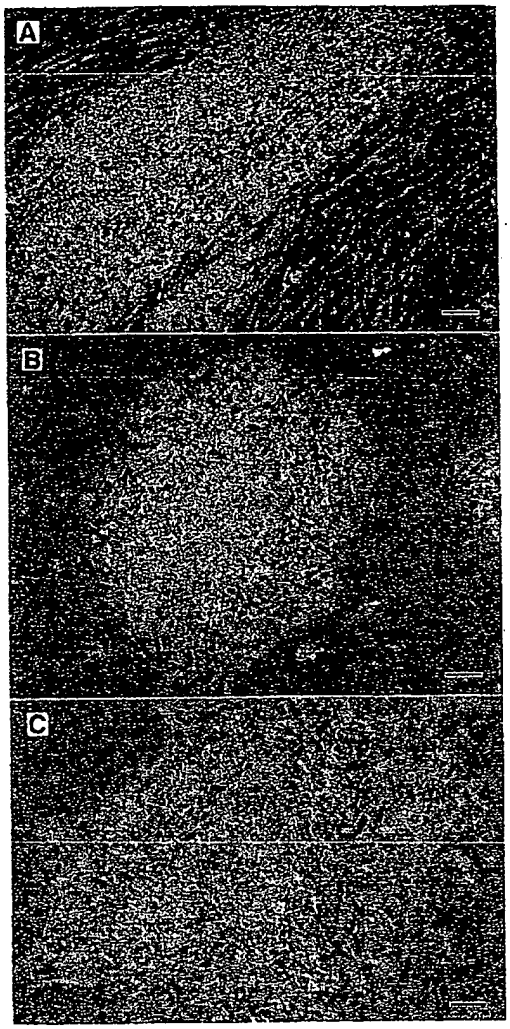

Fig. 4. Human ES cells colonies growing in different culture conditions. **(A)** Human ES cell colony from I-3 line growing 21 passages on mitotically inactivated human foreskin feeder layer (bar = 50 μm); **(B)** human ES cell colony forms I-6 line after several passages on MEF matrix (bar = 75 μm); **(C)** human ES cell colony forms I-6 line after several passages on Matrigel (bar = 75 μm).

Xu et al. demonstrated a culture system in which human ES cells were grown on Matrigel, laminin, or fibronectin using 100% MEF-conditioned medium *(15)*. Richards et al. tested the ability of human feeders or MEF-conditioned media to sustain a continuous undifferentiated proliferation of human ES cells grown on collagen I, human extracellular matrix, Matrigel, or laminin *(14)*. They found that these culture conditions were less suitable for growing human ES cells than with a human feeder layer. In both culture systems, the human ES cells may still be exposed to animal pathogens through the conditioned medium. Another disadvantage of these culture systems is the requirement for the parallel growth of the feeder lines. In our experience, culturing human ES cells on Matrigel or MEF matrix using MEF-conditioned medium is doable but not trivial. We were able to grow ES cells in these conditions for over 15 passages while maintaining all ES cell features. An example of a human ES cell colony growing on a MEF matrix is illustrated in Fig. 5B and on Matrigel in Fig. 5C. The feeder-free culture system for human ES cells still have to be improved in order to be used for the derivation of human ES cell lines. A further discussion on the feeder free culture of human ES cells appears in Chapter 16.

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Expertise:

1. Derivation, differentiation and maintenance of primate embryonic stem cells. Five months advanced studies under the direction of Prof. J. Thomson at the University of Wisconsin, WI, USA (September 1998 – February 1999).

2. Consulting Weill-Cornell University Stem Cells Center on derivation of hESCs in defined conditions. Derivation of WC1 the center first ESC line. NY, USA, August 2005.

3. Consulting INSERM Stem Cells Center on derivation and culture of hESCs. Paris, France, December 2006.

4. Derivation of hESCs lines in defined animal free conditions, in clean rooms, including SOPs preparetion. "Magnet" project, ProChon GMP facility, Rehovot, Israel, 2007-2008.

Research presentations at National and International Meetings:

1. Bar-Ami S., **Amit M.**, Nahir M., and Itskovitz-Eldor J. " γ–Aminobutyric Acid (GABA) induces *in vitro* expansion and hyaluronic acid accumulation in the rat cumulus-oocyte complex". Society for the Study of Reproduction, 30th Annual Meeting, August 1997, Oregon, USA. Vol 56: Abstract 385 pp.179. **Lecture.**

2. **Amit M.**, Margulets V, Segev H, Shariki C, Laevsky I, Coleman R, Itskovitz-Eldor J. "Human feeder layers for human embryonic stem cells". Society for Gynecologic Investigation, 50th Annual Meeting, March 2003, Washington DC, Vol 10 (2); Abstract 746 pp 338a. **Poster**.

3. **Amit M**, Margulets V, Segev H, Shariki K, and Itskovitz-Eldor J. "Alternative culture conditions for human embryonic stem cells". Keystone Symposia "From Stem cells to Therapy" , March-April 2003, Steamboat Spring, Colorado, USA. Abstract 2003 pp 88. **Poster**.

4. **Amit M**, Margulets V, Segev H, Shariki K, Laevsky I, Coleman R, and Itskovitz-Eldor J. "Alternative culture conditions for human embryonic stem cells". The Israeli Society for Infertility Research, Annual Meeting. May 2003, Tel Aviv, Abstract 16. **Lecture**.

5. Suss-Toby E, Gerecht Nir S, **Amit M**, Manor D, and Itskovitz-Eldor J. "Derivation of diploid human pluripotent embryonic stem cell line from mononuclear zygote". The Israeli Society for Infertility Research, Annual Meeting. May 2003, Tel Aviv, Abstract 86. **Poster**.

6. **Amit M**, Margulets V, Shariki K, Segev H, and Itskovitz-Eldor J. "Animal-free culture system for human embryonic stem cells". International Society for Stem Cell Research, Annual Meeting.. June 8-11, 2003, Washington, DC, USA. **Poster** 38.

7. **Amit M**, Margulets V, Shariki K, and Itskovitz-Eldor J. Alternative methods for culturing human embryonic stem cells. NIH Research Meeting: "Recent progress and future promise of human embryonic stem cells". June 12, 2003, Washington, DC, USA. **Poster** 1.

8. **Amit M**, Menke S, Brüning E, Itskovitz-Eldor J, Benvenisty N, Denner J, Winkler M, Martin U. "No evidence for infection of human embryonic stem cells by feeder cell-derived murine leukemia viruses (MLV)". Keystone Symposia " Stem cells", January 2004, Steamboat Spring, Colorado, USA. **Poster 106**.

9. **M Amit**, V Margulets, K Shariki, H Segev, D Manor, and J Itskovitz-Eldor. "Derivation of human embryonic stem cells harboring genetic defects". Keystone Symposia " Stem cells", January 2004, Steamboat Spring, Colorado, USA. **Poster 230**.

10. **M Amit**, K Shariki,V Margulets, and J Itskovitz-Eldor. "Serum and feeder free culture system for human embryonic stem cells". Keystone Symposia " Stem cells", January 2004, Steamboat Spring, Colorado, USA. **Poster 316**.

11. Ulrich M, **Amit M**, Menke S, Bruning E, Winkler M, HaverichA, Itskovitz-Eldor. "No evidence for infection of human embryonic stem cells by feeder cell-derived murine leukemia viruses (MLV)". 2nd International Meeting, Stem Cell Network, North Rhine Westphalia, Germany. **Poster 059**.

12. **M Amit**, V Margulets, K Shariki, and J Itskovitz-Eldor. "Derivation of human embryonic stem cells harboring genetic defects". International Society for Stem Cell Research, 2 th Annual Meeting. June 10-13, 2004, Boston, USA. **Poster 38**.

13. Kristin Schwanke, Monica Winkler, Andreas Schmiedl, Konstantin Miller, **Michal Amit**, Joseph Itskovitz-Eldor, Axel Haverich, Ulrich Martin. Molecular, ultrastructural and immunohistological characterisation of the Rhesus monkey derived embryonic stem cell line 366.4. Keystone Symposia " Stem cells", February 2005, Steamboat Spring, Colorado, USA. **Poster 305**.

14. **M Amit**, J. Chebath, V. Margulets, M. Peri, I. Leavski, Y. Miropolsky, K. Shariki, M. Revel, and J. Itskovitz-Eldor. "Suspended culture of undifferentiated human embryonic stem cells". The international Stem Cell Meeting, June 5-7, 2007, Tel Aviv, Israel.

Lecture.

15. **M Amit**, J. Chebath, V. Margulets, M. Peri, I. Leavski, Y. Miropolsky, K. Shariki, M. Revel, and J. Itskovitz-Eldor. "Suspended culture of undifferentiated human embryonic stem cells", Ilanit - The 5th FISEB Congress Eilat, Israel January 28-29, 2008. **Lecture.**

16. **M Amit**, J. Chebath, V. Margulets, M. Peri, I. Leavski, Y. Miropolsky, K. Shariki, M. Revel, and J. Itskovitz-Eldor. "Suspended culture of undifferentiated human embryonic stem cells", Young researcher day, The international Stem Cell Society, Rehovot, Israel February 21, 2008. **Lecture.**

Invited lectures:

1. "Human embryonic stem cells derivation and spontaneous differentiation." In the course: "News in medical diagnostics" Rambam Medical Center, 6 November 2001.

2. "Human embryonic stem cells: differentiation and clinical uses." Israel Society for the Biology of Aging, Annual Conference, Weizmann Institute of Science, Rehovot, 29 May 2002.

3. "Derivation and differentiation of human embryonic stem cells." Israel Society for Histochemistry and Cytochemistry, Annual Conference, Bar-Ilan University, Ramat Gan, 3 June 2002.

4. Technion Center of Embryonic Stem Cells - Research Report. NIH human embryonic stem cells infrastructure meeting, Washington, D.C., USA June 13, 2003.

5. "Alternative methods for the derivation and culture of human embryonic stem cells." European Society of Human Reproduction and Embryology, Annual Meeting pre-course. Madrid, Spain. June 29, 2003.

6. "Human embryonic stem cells." Israel seminar for high school teachers. Ashdod, 23 March 2004.

7. "Methods for the derivation and culture of human embryonic stem cells." Annual Meeting of the Israel Society for Histochemistry and Cytochemistry. Haifa, 1 June 2004.

8. "From human embryonic stem cells to cloning human beings." Plenary lecture at the Graduation Ceremony. Ort Brauda College of Technology, Karmiel, Israel, 20.5.04.

9. Technion Center of Embryonic Stem Cells - Research Report. NIH human embryonic stem cells infrastructure meeting, Washington, DC, USA June 15-16, 2004.

10. Technion Center of Embryonic Stem Cells – hESCs culture course Report. NIH human embryonic stem cells infrastructure meeting, Washington, D.C., USA. June 15-16, 2004.

11. The second Technion-John Hopkins Symposium in Medical Sciences and Biomedical Engineering. Methods for the derivation and culture of human embryonic stem cells. Haifa,

Israel, October 18, 2004.

12. Rappaport Annual Seminar. Methods for the derivation and culture of human embryonic stem cells. Haifa, Israel, December 30, 2004.

13. "No Evidence for Infection of Human Embryonic Stem Cells by Feeder Cell-derived Murine Leukemia Viruses (MuLV)." Israel Society for Histochemistry and Cytochemistry, Annual Conference, Technion, Faculty of Medicine, Haifa, 1 June 2005.

14. Technion Center of Embryonic Stem Cells - Research Report. NIH human embryonic stem cells infrastructure meeting, Boston, USA June 20-22, 2004.

15. Technion Center of Embryonic Stem Cells – hESCs culture course Report. NIH human embryonic stem cells infrastructure meeting, Boston, USA. June 20-22, 2004.

16. Technion Center of Embryonic Stem Cells - Research Report. NIH human embryonic stem cells infrastructure meeting, San Francisco, USA June 20-22, 2005.

17. Technion Center of Embryonic Stem Cells – hESCs culture course Report. NIH human embryonic stem cells infrastructure meeting, San Francisco, USA. June 20-22, 2005.

18. Technion Center of Embryonic Stem Cells - Research Report. NIH human embryonic stem cells infrastructure meeting, Toronto, Canada June 27-28, 2006.

19. Technion Center of Embryonic Stem Cells – hESCs culture course Report. NIH human embryonic stem cells infrastructure meeting, Toronto, Canada. June 27-28, 2006.

20. Technion Center of Embryonic Stem Cells – Annual meeting. "Suspended culture of undifferentiated human embryonic stem cells", Haifa, Israel December 30, 2007.

21. "Suspended culture of undifferentiated human embryonic stem cells", FISH-ESCs I-STEM , INSERM, Paris, France January31- February 2, 2008.

Awards

The Gutwirth Family Scholarship for Excellence, Technion - Israel Institute of Technology, 2002.

Publications in peer-reviewed journals:

1. Itskovitz-Eldor J, Schuldiner M, Karsenti D, Eden E, Yanuka O, **Amit M**, Soreq H, and Benvenisty N. "Differentiation of human embryonic stem cells into embryoid bodies comprising the three embryonic germ layers". *Mol Med* 6:88-95, 2000.

2. **Amit M**, Carpenter MK, Inokuma MS, Chiu CP, Harris CP, Waknitz MA, Itskovitz-Eldor J, and Thomson JA. "Clonally derived human embryonic stem cells lines maintain pluripotency and proliferative potential for prolonged periods of culture". *Dev Biol* 227:271-278, 2000.

3. Kehat I, Karsenti D, Segev S, Druckmann M, **Amit M**, Gepstein A, Livne A, Binah O, Itskovitz-Eldor J, and Gepstein L. "Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes". *J Clin Invest* 108:407-414, 2001.

4. Assady S., Maor G, **Amit M**, Itskovitz-Eldor J, Skorecki KL, and Tzukerman M. "Insulin production by human embryonic stem cells". *Diabetes* 50:1691-1697, 2001.

5. Levenberg S, Golub JS, **Amit M**, Itskovitz-Eldor J and Langer R. "Endothelial cells derived from human embryonic stem cells". *Proc Nat Acad Sci, USA*, 99: 4391-4396, 2002.

6. **Amit M**, Margulets V, Segev H, Shariki K, Laevsky I, Coleman R, and Itskovitz-Eldor J. "Human feeder layers for human embryonic stem cells". *Biol Reprod* 68: 2150-2156, 2003. First published on line 22 January 2003.

7. **Amit M**, Shariki K, Margulets V, and Itskovitz-Eldor J. "Feeder and serum-free culture system for human embryonic stem cells". *Biol Reprod* 70:837-845, 2004.

8. Suss-Toby E, Gerecht Nir S, **Amit M**, Manor D, and Itskovitz-Eldor J. "Derivation of diploid human pluripotent embryonicstem cell line from mononuclear zygote". *Human Reprod* 19(3):670-5, 2004.

9. Bhattacharya B, Miura T, Brandenberg R, Mejido J, Luo Y, Yang AX, Joshi BH, Irene G, Thies RS, **Amit M**, Lyons I, Condie BG, Iskovitz-Eldor J, Rao MS, Puri RK. "Gene expression in human embryonic stem cell lines: unique molecular signature". *Blood* 15;103(8):2956-64, 2004.

10. Ginis I, Luo Y, Miura T, Thies S, Brandenberger R, Gerecht-Nir S, **Amit M**, Hoke A, Carpenter M, Itskovitz-Eldor J, and Rao M. "Differences between human and mouse embryonic stem cells". *Dev Biol* 15;269(2):360-80, 2004.

11. **Amit M**, Winkler M, Menke S, Bruning E, Itskovitz-Eldor J, Denner J, Ulrich M. "No evidence for infection of human embryonic stem cells by feeder cell-derived murine leukemia viruses (MLV)". *Stem Cells* 23(6):761-71, 2005.

12. Segev H, Kenyagin-Karsenti D, Fishman B, Gerecht-Nir S, Ziskind A, **Amit M**, Coleman R, Itskovitz-Eldor J. Molecular analysis of cardiomyocytes derived from human embryonic stem cells. Dev Growth Differ. 47(5):295-306, 2005.

13. Dolnikov K, Shilkrut M, Zeevi-Levin N, Gerecht-Nir S, **Amit M**, Danon A, Itskovitz-Eldor J, Binah O. Functional properties of human embryonic stem cells-derived cardiomyocytes: Intracellular Ca2+ handling and the role of sarcoplasmic reticulum in the contraction. *Stem Cells*. 24:236-45., 2006.

14. Izrael M, Zhang P, Kaufman R, Shinder V, Ella R, **Amit M**, Itskovitz-Eldor J, Chebath J, Revel M. Human oligodendrocytes derived from embryonic stem cells: Effect of noggin on phenotypic differentiation in vitro and on myelination in vivo. *Mol Cell Neurosci.* 34(3):310-23, 2007.

15. The International Stem Cell Initiative*, Adewumi O, Aflatoonian B, Ahrlund-Richter L, **Amit M**, et al. Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. *Nat Biotechnol.* 25(7):803-16, 2007.

16. Tomescot A, Leschik J, Bellamy V, Dubois G, Messas E, Bruneval P, Desnos M, Hagege AA, **Amit M**, Itskovitz J, Menasche P, Puceat M. Differentiation in vivo of Cardiac Committed Human Embryonic Stem Cells in Post-myocardial Infarcted Rats. *Stem Cells.* 2007 [Epub ahead of print].

17. Aberdam E, Barak E, Rouleau M, de LaForest S, Berrih-Aknin S, Suter DM, Krause KH, **Amit M**, Itskovitz-Eldor J, Aberdam D. A pure population of ectodermal cells derived from human embryonic stem cells. *Stem Cells.* 26(2):440-444, 2008.

18. Hall LL, Byron M, Butler J, Becker KA, Nelson A, **Amit M**, Itskovitz-Eldor J, Stein J, Stein G, Ware C, Lawrence JB. X-inactivation reveals epigenetic anomalies in most hESC but identifies sublines that initiate as expected. *J Cell Physiol.* 2008, in press.

19. GaliaYirme, **Michal Amit**, Ilana Laevsky, Sivan Osenberg, Joseph Itskovitz-Eldor. Establishing a dynamic process for the formation, propagation and differentiation of human embryoid bodies. *Stem Cells and Development*, 2008, in press.

20. Binah O, Dolnikov K, Sadan O, Shilkrut M, Zeevi-Levin N, **Amit M**, Danon A, Itskovitz-Eldor J. Functional and developmental properties of human embryonic stem cells-derived cardiomyocytes. *J Electrocardiol.* 40(6):S192-196, 2007.

21. Brokhman I, Pomp O, Shaham L, Tennenbaum T, **Amit M**, Itskovitz-Eldor J, Goldstein RS. Genetic modification of human embryonic stem cells with adenoviral vectors: differences of infectability between lines and correlation of infectability with expression of the coxsackie and adenovirus receptor. *Stem Cells Dev.* 2008 Jun 13. [Epub ahead of print].

Reviews and Chapters in Books:

1. **Amit M**, Suss-Toby E, Manor D, Itskovitz-Eldor J: Human embryonic stem cells and embryo cloning In: Biotechnology of Human Reproduction. Revelli A, Tur-Kaspa I, Gunar Holte J, Massobrio M (Eds), Parthenon Publishers, London/New-York 2002.

2. **Amit M**, Segev H, Manor D, Itskovitz-Eldor J: Subcloning and alternative methods for the derivation and culture of human embryonic stem cells. In: Human Embryonic Stem Cells. Chiu A, Rao M, O'Grady E (Ed), Humana Press, New Jersey 2003.

3. **Amit M** and Itskovitz-Eldor J: Human embryonic stem cells. In: An Atlas of the Human Blastocyst. Lucinda Veeck, Micika Zaninovic, Nicika Zaninovic (Eds), CRC Parthenon Publishing, London/New York, Chapter 11, pp 213-229, 2003.

4. **Amit M** and Itskovitz-Eldor J. Isolation, characterization and maintenance of primate ES cells. In: Handbook of Stem Cells. Lanze RP (Ed) Elsevier Science, chapter 40, 2004.

5. **Amit M** and Itskovitz-Eldor J: Human embryonic stem cells. In: The Embryo from Conception to Birth: Scientific Discovery, Medical and Ethical Dilemmas. Zimmer, Blazer

(Eds), Karger, Basel, 2003.

6. Izhak Kehat, **Michal Amit**, Amira Gepstein, Irit Huber, Joseph Itskovitz-Eldor, Lior Gepstein. Development of cardiomyocytes from human ES cells. *Methods Enzymol* 365:461-73, 2003.

7. **Amit M**, Gerecht-Nir S, Itskovitz-Eldor J: Derivation, subcloning, spontaneous and controlled differentiation of human embryonic stem cells. In: Stem cells: from bench to bedside, Bongso and Lee (Eds), World Scientific Publishing, Singapore, 2005.

8. **Amit M**, Itskovitz-Eldor J: Isolation, characterization and maintenance of primate ES cells. In: Essentials of Stem Cell Biology, Lanza RP (Ed), Elsevier Science, 2005.

9. **Amit M**, Itskovitz-Eldor J: Derivation and maintenance of human embryonic stem cells. In: Human embryonic stem cells:methods and protocols, Turksen (Ed), Humana Press, Canada 2005.

10. **Amit M**, Itskovitz-Eldor J: Maintenance of human embryonic stem cells in animal-serum- and feeder-layer-free culture conditions. In: Human embryonic stem cells:methods and protocols, Turksen (Ed), Humana Press, Canada 2005.

11. **Amit M**. Feeder layer free culture system for human embryonic stem cells. In Methods in Molecular Biology 407, Stem Cells Assays, Mohan C Vemuri (Ed), Humana Press, Chapter 2 pp. 11-20, 2007.

12. **Amit M**, Itskovitz-Eldor J. Feeder-free culture of human embryonic stem cells. *Methods Enzymol.* 420:37-49, 2006.

13. **Amit M**, Itskovitz-Eldor J. Maintenance of human embryonic stem cells in animal serum- and feeder layer-free culture conditions. *Methods Mol Biol.* 331:105-13, 2006.

14. **Amit M**, Itskovitz-Eldor J. Derivation and maintenance of human embryonic stem cells. *Methods Mol Biol.* 331:43-53, 2006.

15. **Amit M** and Itskovitz-Eldor J. "Derivation and spontaneous differentiation of human embryonic stem cells. Review." *J Anat* 200:225-232, 2002.

16. **Amit M**, Itskovitz-Eldor J. Sources, derivation, and culture of human embryonic stem cells. *Semin Reprod Med.* 24(5):298-303, 2006. Review.

17. **Amit M**. Derivation of stem cells from epiblasts. Emerging technology platforms for stem cells, Editors: Uma Lakshmipathy, Jonathan D Chesnut, and Bhaskar Thyagarajan, Wiley Publishers, 2008 in press.

Teaching:

1. Ort Brauda College of Technology, Karmiel, Israel, supervisor

Final apprenticeship for B.Sci. degree in Biotechnology:

Yael Miropolski 2004.

Topic: "Optimization of the feeder layer free culture of hESCs: suggested model for the cellular transduction mechanism".

2. Ort Brauda College of Technology, Karmiel, Israel, supervisor
Final apprenticeship for B.Sci. degree in Biotechnology:

Lina Kalish 2005.

Topic:"The effect of bFGF from different sources on the karyotypes of cultured hESCs".

3. Technion, Faculty of Medicine,
M.Sc degree in Medicine Science, advisor:

Liat Aharon 2005-08.

Topic:" Differentiation of human embryonic stem cells into retinal cells".

4. Technion, Faculty of Medicine,
M.Sc degree in Medicine Science, advisor:

Atara Patrero 2005-08.

Topic:" Proteomics and Molecular Profiling of Human Embryonic Stem Cells in Early Differentiation Stages".

5. Technion, Faculty of Medicine,
M.Sc degree in Medicine Science, advisor:

Hadas Bookay 2005-08.

Topic:"Magnetic Cell Sorting Purification of Differentiated Human Embryonic Stem Cells Stably Expressing specific Surface Marker".

6. Technion, Faculty of Medicine,
M.Sc degree in Medicine Science, advisor:

Hagit Domev 2006-08.

Topic:" Fluorescence Cell Sorting of Skeletal Myoblasts Derived from Human Embryonic Stem Cells".

7. Technion, Faculty of Medicine, supervisor
M.D degree in Medicine, basic science:

Liron Eldor 2005-07.

Topic:" Human embryonic stem cells differentiation to epithelial tissue".

8. Technion, Faculty of Medicine, supervisor
M.D degree in Medicine, basic science:

Yekaterina Shlush 2004-08.

Topic:" Human embryonic stem cells induced differentiation to stratified or smooth muscle".